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HYDRODICTYON: A COMPARATIVE BIOLOGICAL
STUDY.

By M. A. Pocock.

(With Plates XII—XX.)

- I. *Hydrodictyon patenaeforme* Pocock.
II. *Hydrodictyon reticulatum* (L.) Lagerheim.
III. *Hydrodictyon africanum* Yamanouchi.

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ABSTRACT.

Prolonged field studies of these three species of *Hydrodictyon* have been made, supplemented by extensive cultures. All are amenable to cultural methods, *H. reticulatum* outstandingly so. In all there is great plasticity, response to external conditions being immediate and reversible. The use of agar-plate cultures has made it possible to follow the fate of individual cells and coenocytes.

In all the BASIC STRUCTURE is a network of coenocytes, each containing many nuclei and conspicuous pyrenoids; in *H. reticulatum* and *H. patenaeforme* the coenocyte is cylindrical, but in *H. africanum* after passing through a cylindrical stage it begins to swell up, becomes barrel-shaped and finally spherical, the coenocytes dissociating as this happens.

The CHLOROPLAST in the well nourished maturing coenocyte is a fenestrated parietal sheet, lining the whole wall. Partial starvation causes the fenestrations to extend, the chloroplast becomes extensively vacuolated, then reticulate and eventually granular, but on return to favourable conditions recovery may be complete.

DIVISION OF THE PROTOPLAST preceding reproduction follows the same sequence in all three:—preparation, involving multiplication of nuclei, disappearance of pyrenoids, division of the chloroplast into small rounded granules and rearrangement of the latter round each nucleus, culminates in the PAVEMENT STAGE finally followed by cleavage of the protoplast. Each segment thus formed becomes a uninucleate motile cell.

REPRODUCTIVE CELLS are of four categories, all, except the planozygote and occasionally zoospores, biflagellate: (1) ZOIDS formed in vast quantities in coenocytes of the net and in smaller numbers by the polyhedron, or in *H. africanum* by direct germination of the hypnospor. Zooids may either (a) come to rest without liberation and reunite to form a daughter net (*H. reticulatum*) or a germ net (all three species), or (b) be liberated and after a period of motility settle down without conjugating to form haploid (azygotic) hypnospor, occasional in *H. reticulatum*, the usual procedure in *H. patenaeforme*, and possibly also in *H. africanum*; (2) GAMETES which after liberation swarm and conjugate forming zygotes. In *H. reticulatum* gametes are formed in even greater numbers than zooids, are about half their size and very active; in *H. patenaeforme* they are apparently indistinguishable from zooids and possibly all are facultative gametes. Both these species are isogamous and sometimes at any rate homothallic. *H. africanum* on the other hand is anisogamous; (3) the PLANOZYGOTE resulting from conjugation of two gametes is quadri-flagellate, remains motile for a time then settles down to form the zygospore (diploid hypnospor); (4) ZOOSPORES, comparatively large, rather slow moving biflagellate cells, formed on germination of hypnospor, one from each azygotic hypnospor, typically four, sometimes eight from each zygospore; in the latter other numbers, from one to seven, may occasionally result, in which case one or more may have more than one nucleus and associated pair of flagella.

HYPNOSPORES, no matter what their origin, are similar in appearance and development; in *H. reticulatum* and *H. patenaeforme* on germination they produce zoospores which in a very short time settle down to form the POLYHEDRON, an interpolated growth-phase ending in the formation of the germ net; in *H. africanum* polyhedra have occasionally been seen but normally germination is direct, the germinating hypnospor itself giving rise to the germ net.

THE GERM NET may be completely flat in all three species, but whereas such flat nets are normal in *H. patenaeforme* and *H. africanum*, usually composed of 512, 1,024 or more coenocytes, in *H. reticulatum* flat nets are rare, few celled (64,128) and the normal form is a sac-shaped net of some 256 coenocytes.

THE DURATION OF THE GERM NET in *H. reticulatum* is short; each coenocyte soon produces a daughter net of several thousand coenocytes, each of which in turn may give rise to a daughter net. The daughter nets are all of the form of the coenocyte, i.e., a cylinder with closed ends. In the other two species no daughter nets are formed, the germ net is the only net in the life cycle, is long lived and attains a comparatively large size before eventually producing swimmers, whether gametes, facultative gametes, or zooids.

The TIME taken from liberation of swimmers to formation of the germ net depends largely on external conditions; in culture it may be as short as 10 (*H. africanum*), or 12-13 days (*H. reticulatum* and *H. patenaeforme*).

The LIFE CYCLE throughout is marked by recurring periods of growth with multiplication of nuclei and pyrenoids (in the coenocyte, hypnospor and polyhedron), alternating with the production of motile cells, preceded by the disappearance of pyrenoids.

MOTILE CELLS, no matter of what category, may on occasion come to rest and form either isolated coenocytes or resting cells which develop according to the species concerned. Various other abnormalities are recorded.

INTRODUCTION.

The Water Net, *Hydrodictyon reticulatum* (L.) Lagerheim, is in many ways unique, standing out from among its nearest relations, and indeed from among all algae, by virtue of its striking yet in some ways simple construction as well as its remarkable form of asexual or vegetative reproduction which, in a very short space of time, may result in a huge population of cylindrical green nets.

Until comparatively recently the genus *Hydrodictyon* was regarded as monotypic but the description of two species from South Africa—*H. africanum* Yamanouchi (1913) and *H. patenaeforme* Pocock (1937)—while extending the limits of the genus has also necessitated a revision of the old-established conception thereof, since the study of their life history shows that characters previously regarded as generic are specific only, occurring in the original species but not in the two new African species.*

In *H. reticulatum* two types of reproduction have long been recognized. Of these, the better known and more striking is the very characteristic form of asexual reproduction. Here the protoplast of each constituent coenocyte divides into a large number of uninucleate zooids (macrogonidia of earlier investigators, cf. Braun, Cohn, Rabenhorst, etc.); after a brief period of movement of a peculiar and very restricted type, the nature of which has given rise to considerable divergence of opinion, these zooids, without being liberated, rearrange themselves and unite to form a new net inside the parent coenocyte. Since the protoplast enclosing the large central vacuole lines the wall of the coenocyte and retains this position throughout the whole process of division, differentiation, separation and subsequent re-union of the zooids, it follows that the daughter nets necessarily assume the form of the parent coenocyte; hence the daughter nets produced by normal well-developed nets are typically

* *Hydrodictyon indicum* Iyengar (1925), founded on a single isolated record from near Madras, remains imperfectly known (cf. Pocock 1937, p. 278) and is therefore not considered here.

cylindrical but with closed ends (Pl. XII, A. B). So predominant are these elongated sac-shaped nets in the life history of this species, that their form has generally been accepted as that characteristic not only of the species,* as is indeed implicit in the specific name "utriculatum" given by Roth (1797, p. 48) and still in very general use among continental botanists, but also of the genus (cf. Rabenhorst, 1868, p. 65).

In the case of the two South African species, however, prolonged study both in the field and in culture shows that in them this type of reproduction is unknown, the net being fundamentally plate-shaped (Yamanouchi, p. 74; Pocock, p. 264) and cylindrical nets never occurring.

In the second type of reproduction the protoplast divides into a larger number of smaller swarmers (microgonidia of the earlier workers) which are liberated, swarm freely in the water and there conjugate to form planozygotes. After a short period of activity, the planozygote comes to rest, withdraws its flagella, rounds off and secretes a wall, forming a minute resting spore. The sexual nature of these swarmers was for a long time not recognized, although from Braun's statement that they had two flagella "but sometimes three or four" it is probable that he had observed planozygotes among the swarming gametes. Rabenhorst (Fig. 35) figures quadriflagellate as well as biflagellate swarmers. Pringsheim (1860) studied the development of the resting spores and although he too failed to recognize their asexual origin, he successfully followed them through a period of quiescence, succeeded by one of growth which culminated in germination when the contents divided, most often into four parts, giving rise to four rather large zoospores; but he also observed cases where two, three or five zoospores were formed from one resting spore (i.e., p. 81). The zoospore in turn settled down to form an angular

* For example: Braun (p. 62) "... sistens ret saccatum, oblongum, undique clausum". Wood (p. 92) "... beautiful cylindrical nets". Klebs (1896, p. 133) "*Hydrodictyon* erscheint im Form Langer schlauchformiger geschlossener Netze". West (p. 297) "... a more or less cylindrical net". Smith (p. 486) "cells ... are united to form a sac-like reticulum". Fritsch (p. 171) "The coenobium in this case is a free-floating hollow cylindrical network closed at either end".

PLATE XII. *Hydrodictyon reticulatum*.

A, E—G, from Grahamstown. B—D, from Cambridge.

- A. Part of a net on agar in process of daughter-net formation(964). $\times 27$.
 - B. Portions of two well-formed cylindrical nets (on agar) somewhat thrown into folds, one still enclosed in remains of wall(924). $\times 37$.
 - C. Nets of various shapes and ages, some few-celled(807). $\times 25$.
 - D. Part of C (top right) enlarged to show detail of structure and remains of mucilaginous inner wall round small net(808). $\times 160$.
 - E. Gametes swarming. Flagella slightly retouched(1013). $\times 1100$.
 - F. Germinating zygospore. First cleavage complete, second incipient. Two zoospores subsequently liberated(968). $\times 375$.
 - G. Zoospore just liberated from azygotic hypnospore(969). $\times 375$.
- N.B.—The number in brackets is the serial number of the negative reproduced.



PLATE XII. *Hydrodictyon reticulatum*.

body to which he gave the name "Polyeder". Again a period of growth was followed by division of the protoplast as in asexual propagation and a young net, the "germ" net, was formed.

Rostafinski (1875, p. 152), discussing a possible natural classification of "les algues chlorosporées" based on the type of sexual reproduction, states that in 1873 Suppanetz, working in De Bary's laboratory, discovered copulation of the "microzoospores" of *Hydrodictyon*, observing it sometimes even within the parent cell, or soon after escape. But besides the normal conjugation in pairs, he noted copulation of three or even six, hence either he was misled by the "clumping" which precedes copulation or else he was dealing with abnormal material. The "isospore" formed by copulation developed as described by Pringsheim.

Klebs (1890, p. 353), who also refers to these observations of Suppanetz, like Rostafinski fully accepts the sexual nature of the swarmer, applying to it Strasburger's recently proposed term *gamete*, and in this is followed by succeeding algologists.

No reference is found in the literature to any other type of free motile cell in the life history of *Hydrodictyon reticulatum* and apart from these two modes of reproduction no other method of propagation has so far been recognized in the genus, no accessory spores being known. Thus, since daughter-net formation is unknown in the two South African species,* it seemed as though these two species must depend for their propagation entirely on sexual reproduction.

In *H. patenaeforme* swarming and conjugation of gametes and the rounding off of resting spores had been seen on several occasions (Pocock, p. 274); further, polyhedra and the germ nets formed from them had also frequently been observed, but the stages intervening between the rounding off of the planozygote to form the resting spore and the formation of the polyhedron were still unknown. It was to be expected that development would follow the same lines as in *H. reticulatum* and the attempt to verify this expectation initiated the series of cultural experiments described here. The somewhat startling results of the first cultures led to further investigations of both this and the other two species and it became apparent that the life history of *Hydrodictyon* is far from being the stereotyped, restricted story hitherto accepted as characteristic of the genus; on the contrary, it is highly plastic, susceptible of a number of variations.

In the present paper three species of *Hydrodictyon* are considered: *H. patenaeforme* and *H. reticulatum* are treated in considerable detail;

* In the case of *H. patenaeforme* the absence of daughter-net formation has recently been noted in material obtained from near Lake Titicaca in Peru (Tutin, 1940, p. 197).

H. africanum presents certain individual problems which have not yet been fully solved, but since repeated experiments made at various times have yielded similar results, an account of the work so far done on this species is included, although obviously much more is required to elucidate fully the curious divergences from the two former species noted at several stages in the life history of this most striking species of the genus.

Special attention has been given to the mechanism of net formation both in the daughter net of *H. reticulatum* and in the germ net of all three species.

SOURCES OF MATERIAL.

The material used in the investigation has been obtained from a number of localities, the chief of which are described here:

I. *Hydrodictyon patenaeforme*.

1. *Groen Vlei* and *De Klip* on the Cape Flats.

Groen Vlei, on the Lansdowne Road, Claremont, Cape Province, is an extensive grassy field on alluvial soil, several acres in extent, inundated more or less completely for several months during the wet season. In summer it is used as grazing ground for cattle and is therefore heavily charged with organic matter. When under water it is frequented by numbers of wading birds—Hérons, Egrets, Stilts, etc., occasionally even Pelicans. Parts of the vlei are annually filled with great masses of *Hydrodictyon patenaeforme*, the young nets of which develop in vast numbers on or just above the floor of the vlei, floating upwards as they enlarge, while more and more develop below them until the water is filled with nets in all stages of development, from minute delicate green nets below, through increasing sizes of bright green, larger-meshed nets until the upper layers are reached, where the largest are found. As the season advances, under exposure to the strong sunlight the nets in the upper layers develop haematochrome and so great is the bulk of nets that at a little distance the surface of the water appears a bright reddish orange. In the same vlei, besides a large species of *Aedogonium* and one of the largest of *Spirogyra*, *Sphaeroplea wilmanae* is also abundant; like *Hydrodictyon* it too forms brightly coloured patches in the vlei, but more nearly flame-coloured, redder in tone than the latter, so that at this stage it is possible at a glance to distinguish areas in which *Hydrodictyon* is abundant from those in which *Sphaeroplea* predominates. In the latter, the haematochrome is however not formed in the vegetative cells but in the ripening oospores which crowd the cells of the female filaments.

At De Klip one of the several small pools which used to form in winter alongside the granite bosses produced each year a large crop of *Hydrodictyon patenaeforme*, which eventually completely filled the water of the narrow pool; this happened regularly every year from the time when the pool first came under observation nearly 30 years ago. A second pool a few yards away used also to produce *Hydrodictyon*, but after a few years ceased to do so.* In a third pool, the "Peridinium" pool, on white sand adjacent to the granite near "Port Jackson willow" (*Acacia* sp.) bushes, *Hydrodictyon* had never been found until in the spring of 1940 portions of nets and abnormally large isolated coenocytes, up to 6 cm. long by 2 mm. in diameter, were collected at the deep shady end of the pool among *Nitella* plants. Soil from the floor of the first pool when dry was used repeatedly for cultures which nearly always yielded nets which have been used repeatedly in the laboratory as the source for swarmers, as well as the nets formed in the pool. In this pool too polyhedra and germ nets were repeatedly found, early in the season soon after the pool had formed. Nearby, roadside ditches in which rainwater collects have also yielded good material of this species.

2. *Grahamstown* (Albany Division of the eastern region of the Cape Province).

Hydrodictyon patenaeforme is of common occurrence in a number of localities in the neighbourhood of Grahamstown. During the period February to June 1938, following an exceptionally good rainy season, it occurred in quantity in pools of the Palmiet River, a small tributary of the New Year's River; it was particularly abundant in a series of large pools below the dam on the farm Burnt Kraal, three miles from the town. These pools are fairly deep (1 to 2 or 3 ft.) and partially shaded by small trees and bushes and the nets were remarkable both for their retention of green colour and for the large size attained with hardly any twisting of the coenocytes, in these respects differing markedly from the Cape Flats material. One net examined was still flat although it measured some 20 cm. in diameter with coenocytes 8—10 mm. long. In some years it was also abundant in pools lower down the Palmiet near its junction with the New Year's River on Table Hill Farm.

The early part of 1939 was also a good season for *Hydrodictyon* in this region and Miss Britten reported it from several localities. Material raised from swarmers produced by coenocytes received from Miss Britten (see below) provided cultures which have been used repeatedly.

* When full, most of the De Klip pools were used as a laundry by the local population—a very poor one—and although many of the algae seemed able to survive a diet of soapy water, the vegetation as a whole suffered. The first *Hydrodictyon* pool being shallow was one of the least used for this purpose. Unfortunately, since the above was written extensive quarrying operations have destroyed nearly the whole of De Klip.

II. *Hydrodictyon reticulatum*.

1. *From England.*

Material obtained from the Botany School, Cambridge, in 1936 has been kept in culture for some years, first in Chelsea, then from 1937 until 1948 in South Africa.* The species is ideal for cultural purposes as it is extremely tolerant of varying conditions.

2. *From South Africa.*

In the wild state *H. reticulatum* had never been reported from South Africa, where the commonest species is *H. patenaeforme* in which, as mentioned above, daughter-net formation does not occur. When, therefore, early in March 1939 Miss Britten reported the discovery of daughter-net formation in material collected on the farm Heatherton Towers, 20 miles from Grahamstown, considerable interest was aroused. The question as to the species concerned at once arose—was this the long-sought asexual reproduction in *H. patenaeforme* or had Miss Britten established a new record for *H. reticulatum*? The nets had been collected in a furrow leading water through the farm from a dam on the Botha's River near its junction with the Fish River. When first examined the majority of the nets were large meshed with stout coenocytes about 6–10 mm. long, tangled and liable to break apart. But among these were smaller nets the constituent coenocytes of which were much shorter and more slender. Material kept in the laboratory and examined two days later was found to be actively reproductive, the larger meshed nets producing swarmers, while those with the smaller coenocytes were forming daughter nets. On a subsequent visit (20th April) no nets of the first type were found, but there were numbers of long cylindrical nets, presumably either the daughter nets which were being formed on the earlier visit or their progeny.

Fortunately, realizing the interest of the discovery, Miss Britten reported it at once by telegraph, and in response to a request (also by telegraph) for living material, was able to send some by air mail. This material was packed in tubes plugged with cotton wool after the free water had been drained off, and in the moist atmosphere of the tubes the coenocytes, although in the case of the larger nets mostly disjunct, carried well, arriving in Rondebosch on the 10th March healthy and normal despite their 600-mile aerial journey. On arrival, many of the larger

* No details as to its origin could be learnt, except that it had been in culture in the Cambridge Botany School for a number of years.

coenocytes were already full of active swimmers while others were about to divide, and on transference to culture solution liberation at once began. The swimmers showed no trace of abnormality, were very active but showed no sign of conjugating. Mixed with these large coenocytes were fragments of nets with much smaller bright green coenocytes; these were isolated in culture solution and in the course of a few days produced beautiful cylindrical daughter nets.

Five weeks after liberation of the swimmers several tiny flat nets 3—4 mm. in diameter were noticed in beakers of culture solution into which some of the swimmers had been put. These were obviously germ nets from spores which had germinated and when first seen were already several days old; it followed that the resting period, if any, of the spores must have been brief.

Isolated in fresh culture solution these nets developed as typical *H. patenaeforme*, showed no sign of daughter-net formation and ultimately formed swimmers. Resting spores resulting from these nets as well as from the swarming coenocytes first received have been germinated, always with the same results, and at the time of writing (1941) nets of the ninth or tenth generation have been raised, propagation taking place exclusively by means of liberated swimmers, never by daughter nets.

The cylindrical nets obtained from the smaller coenocytes on the other hand developed as typical *H. reticulatum*, reproducing freely by both daughter-net formation and by swimmers. Their progeny has now been carried to at least the fifteenth generation by daughter-net formation, side by side with the same species from Cambridge. This by no means represents the possible number of asexually produced generations obtainable in the time; cultures have been allowed to stand for weeks under conditions which precluded the formation of reproductive bodies. This is specially true as regards the formation of daughter nets. By continual culture of isolated nets in abundance of culture solution, a very much larger number of generations could have been obtained in the time. At the same time several, though fewer, generations have been obtained by sexual reproduction.

Obviously, then, Miss Britten's original material was a mixture of the two species *H. reticulatum* and *H. patenaeforme*. The latter, being mature, produced swimmers and disappeared, while the former produced daughter nets which formed the water-net population of the furrow on the second visit. This constitutes a new record for *H. reticulatum* since, although widely distributed in North Africa, for example in Algeria (Gauthier-Lièvre, pp. 23, 244) and Egypt (Nayal, p. 20) it has not yet been reported

in South Africa;* the "*Hydrodictyon reticulatum*" recorded and figured by Marloth (1913, I, p. 10) is obviously not this species but *H. africanum*, subsequently described by Yamanouchi.

In Egypt, as in China, *H. reticulatum* occurs in quantity in paddy fields where beautiful large nets, some 20—30 cm. long, were collected by Professor Oliver and sent by him to Professor Fritsch. The Grahamstown habitat is thus a very different one and the occurrence there of the two species is of interest ecologically, affording one more instance of the extraordinarily diversified character of the flora of the Albany Division, a region which is the meeting place of several distinct types of flora; what is true of the flowering plants is reflected also in the algal flora so far as this is known, types characteristic of regions of both summer and winter rainfall being present.

3. From America.

Material collected at Cherry Creek, Nevada County, California (Nobbs), in 1949 has been kept in culture, first at Cape Town, subsequently at Grahamstown for 10 years and has provided additional information as to the behaviour of this species under varying conditions of culture.

III. *Hydrodictyon africanum*.

All the material of this species used in the investigation was obtained either directly from vleis on the Cape Flats, chiefly a number on the Klipfontein Road beyond Athlone and Isoetes Vlei on the Lansdowne Road, beyond Claremont, or from cultures of soil from the latter locality.

It is by far the rarest species; so far as is known entirely restricted to the coastal region extending from about the centre of the Cape Flats northwards to some hundred miles north of Cape Town.

* In 1938 part of the Cambridge material was taken to Grahamstown and kept in culture in the Botany Department of Rhodes University. There it thrived and from time to time part of the progeny was put into the small cement pool (about 3 x 6 ft.) in the quadrangle. But in the open it never succeeded, and each time disappeared almost immediately from the pool. Nevertheless, the possibility that the Heatherton Towers material might derive from this was considered. In view of all the circumstances, however, in particular the distance between the two places and the fact that the algae had not flourished in the open, this possibility has finally been rejected. Further, Miss Britten has since (June 1939) collected it in a second locality—in backwaters of the Fish River at Committees, some distance from Heatherton Towers. Here it was associated with *Enteromorpha*, *Sphaeroplea*, *Cladophora* and *Spirogyra*. Recently, Professor Omer-Cooper has suggested the possibility of dissemination of the resting spores by water beetles, thus opening up a most interesting field of investigation.

METHODS OF CULTURE.

Two methods of culture used in conjunction with one another have proved most useful, viz. liquid media and agar-plate culture.

1. *Liquid Media.*

At first Beneke's Solution (half strength) plus 10 per cent soil decoction was used, but this was soon discarded in favour of the culture solution recommended by Juller (1937, p. 61) and called by him "Volvox solution" combined with soil extract. This is such a valuable nutrient solution for general culture work that its composition is repeated here with such modifications as proved useful in practice:

Potassium nitrate, Magnesium sulphate, Secondary

Potassium phosphate	..	..	..	0.05 gr. each
Potassium carbonate	..	..	..	0.07 gr.
Calcium nitrate	..	..	..	0.20 gr.
Ferric chloride	..	..	..	0.01 gr. (or less)
Soil decoction	..	..	..	300 cc.
Glass distilled water	..	..	..	3000 cc.

When ready for use the solution is approximately neutral with a pH concentration of from 7—7.3.

For convenience in actual practice stock solutions of the six salts are made up separately in small flasks, each in 100 cc. glass distilled water: Potassium nitrate, Magnesium sulphate, Potassium phosphate each 5 gr., Potassium carbonate 7 gr., Calcium nitrate 20 gr., Ferric chloride 1 gr. As culture solution is needed, to 3000 cc. glass distilled water in a pyrex flask, 1 cc. of each of the stock solutions is added plus 300 cc. freshly made soil decoction.

An adaptation of the method introduced by Pringsheim (1926, p. 286; cf. also Mainx, 1928, p. 323) was used in preparing the soil decoction. Approximately equal volumes of soil and glass distilled water in a flask are brought to the boil in a steam sterilizer, allowed to stand overnight and the supernatant liquor then decanted off, being strained through fine muslin to get rid of floating organic fragments. This is again allowed to stand so that fine particles in suspension separate out, collecting on the floor of the flask, leaving a clear brown or amber liquid (according to the amount of organic matter present) which is again decanted. In this some bacterial action generally followed and it was usually strained again before use. As bacteria-free cultures were not attempted, further sterilization was usually omitted; for pure culture work the means of sterilization available at the time were inadequate.

At first garden soil was used in preparing the soil extract; later this was replaced or supplemented by soil collected in the dry season from the depressions in which rain later collects to form the pools or vleis in which the algae to be cultured grow. Thus the soil decoction most suited to the alga in question is obtained. Since such vlei soils are usually heavily charged with organic matter, both plant and animal, in preparing the soil decoction a larger proportion of water (about 2: 1) was generally used.

Both *Hydrodictyon reticulatum* and *H. patenaeforme* thrive in the culture solution prepared in this way; vegetative development was fully normal, nets developing at least as well as in optimum field conditions while in the former species daughter nets formed freely. But that it may nevertheless not be absolutely ideal is suggested by a reluctance in both species to reproduce sexually, conjugation being comparatively rare. This may however perhaps be attributable to other concomitant circumstances, such as temperature or incidence of direct sunlight, and not to the nature of the nutrient fluid. That this may have been the case is suggested by the behaviour of *H. reticulatum* during the 1940-41 experiments: whereas in the winter and spring asexual reproduction predominated, in the later experiments (December, January), when the temperature was considerably higher, true gametes which conjugated readily even before liberation, were formed more commonly than daughter nets. Much still remains to be learnt as to the effect of external conditions on the behaviour of both species.

In the case of *H. africanum* it was found that normal development was not obtained in culture solution unless at least part of the soil used in making the soil extract was from a vlei in which this species occurs. With this modification, the same culture solution gave excellent results (cf. net shown in Plate XX, J).

2. Agar Plates.

Various concentrations of agar, 0.5, 0.75 and 1 per cent made up in the above nutrient solution instead of water, were tried; on the whole, 0.75 per cent proved the best for the present investigation.

Plate cultures proved specially useful for watching the development of spores and changes in the coenocyte, particularly in daughter-net formation in *H. reticulatum* (cf. Pls. XVII, C-E, XVIII, B-G), and in swarmer formation in all three species. Not only is it possible to exercise more control than in liquid media, but in addition the agar itself seems to stimulate development.

The chief drawback to the use of agar plates is that other organisms also thrive on them. In some cases, particularly when material direct from vleis was used, other green algae were introduced on to the plates

where they, too, flourished. In general, however, resting spores of *Hydrodictyon* are easily distinguishable from other organisms.

INVESTIGATION OF THE SPECIES.

I. HYDRODICTYON PATENAEFORME.

A. INITIAL EXPERIMENTS.

1. Material from Groen Vlei.

In the afternoon of Saturday, 20th November 1937, *H. patenaeforme* was collected at Groen Vlei in a small rather deep pool near the road—all that remained of the extensive vlei of a few weeks earlier. The nets were old, large and tangled, consisting mostly of very large coenocytes (up to 2 cm. long) and nearly all yellowish green or golden in colour. Part of the material was put in a vasculum, part in jars containing a little water. The following morning it was transferred to flat glass dishes of fresh tap water. About noon on Monday it was found that swarmer (Fig. 1, a, b) were being liberated in vast numbers. Before long the water was opaque with myriads of swarmer which soon began to congregate on the side of the glass towards the light.

Numerous samples were examined under the microscope. Movement was extremely energetic and the tendency to form dense clusters or "clumps" as described by Mainx (1931, p. 506), in the swarmer of *H. reticulatum* very marked. But in every case if one such clump were watched it was found that after a short time, usually a few seconds, the swarmer dispersed in all directions, almost as if some sudden mutual repulsion had come into play. Further, again and again swarmer were seen to come together in pairs and move about one another as if about to copulate, but, instead of union being effected, after a brief association the two swarmer separated and moved away from one another. From time to time drops of water containing swarmer were treated with iodine and examined for quadriflagellate planozygotes but not one was seen, and, apart from the temporary association, no sign of conjugation in either living or fixed material could be detected, even after prolonged swarming.

This was in marked contrast to the behaviour of gametes observed in the Kimberley material (Pocock, p. 274) and on several occasions in material from the Cape Flats. In these cases conjugation proceeded actively and the phenomena accompanying it, in particular the peculiar movement of the conjugating pairs, were most obvious even under low powers of magnification. The differences in behaviour could hardly be attributable to lowered vitality of the swarmer since they appeared to be just as active and energetic in their movement in the non-conjugating Groen Vlei material as in that in which conjugation was taking place.



FIG. 1.—*H. patenaeforme*. Asexual Reproduction. (i) Formation of azygotic hypnospore: a, b, asexual swimmers; c—e, development of young hypnospore: c, newly formed; d, enlarging, one pyrenoid; e, two pyrenoids present, wall still thin; f, mature azygospore ready for germination. (ii) Germination and formation of polyhedron: g, endospore thickening at anterior pole; h, j, exospore ruptured, endospore protruding; k—p, stages in germination of a single spore: k (2.45 p.m.), vesicle formed, zoospore ready to escape; l (2.55 p.m.), escape of zoospore; m (3 p.m.), zoospore coming to rest, one posterior protrusion; n (3.1 p.m.), flagella withdrawn, numerous "pseudopodia" formed; o (3.45 p.m.), wall forming, some protoplasmic processes fixed as spines, others still metabolic; p (5 p.m.), polyhedron fully formed. q, polyhedron formed inside vesicle. r—s, germinating azygospores showing inverted polarity.

a—e $\times 1500$; other figures $\times 500$.

Formation and Development of Resting Spores. Water from the side of the dishes nearest the light where the swimmers were concentrated was pipetted off, some put in small beakers (with or without added culture solution), and some on agar plates. On each plate several drops were placed, each sufficiently large for movement of the swimmers in it to continue freely for some time before the water disappeared into the agar. The majority of the swimmers quickly collected on the side of the drop towards the light, a smaller number congregated on the opposite side where light was focused through the drop, while a few were scattered along the rest of the circumference and over the surface of the drop.

This was the distribution when movement eventually ceased. Each swimmer then rounded off, secreting a delicate smooth hyaline wall (Fig. 1, c); in this case, since the parent nets were golden in colour, the swimmers and the minute spherical resting spores were also golden (cf. Pocock, pp. 272 and 274). The glass containers and the agar plates were left in a south window where, although well lighted, no direct sunlight reached them.

At the end of a week the resting spores in the beakers to which no nutrient solution had been added were still minute ($6-8\mu$) and golden in colour, i.e. much as when first formed, whereas those which had been supplied with nutrient solution, whether in liquid or on agar, were enlarging and had turned bright green. Since the agar plates were the most easily observed, attention was concentrated on them with occasional examination of material from the liquid media; addition of culture solution to the beaker containing the small golden spores at once resulted in the commencement of growth accompanied by a colour change to green.

It is easy to examine spores on agar without disturbing them; with care, the ordinary high-power objective can be used directly without a cover slip. If a small drop of water is added, the water-immersion lens can be used, or a cover slip can be put on the agar and the oil-immersion lens used without shifting or injuring the spores, even when the cover slip is removed. For detail of spore structure one or other of the immersion lenses must be used.

When first formed the chloroplast resembled that of the swimmer—usually granular without a pyrenoid, but sometimes already continuous with a small pyrenoid, the eyespot conspicuous. As growth began and the wall thickened the eyespot disappeared and the chloroplast became homogeneous, forming a slightly vacuolated parietal layer more or less completely lining the wall, thickened at one side where the pyrenoid was embedded (Fig. 1, d), while the nucleus with its conspicuous nucleolus lay either centrally or more rarely to one side. As in the Volvocales, the nucleus is large and can be seen even when living and unstained (Pl. XIII,

C, and Fig. 1, c—e). Later a second smaller pyrenoid sometimes made its appearance, usually on the side of the spore opposite to the original pyrenoid (Pl. XIII, D, and Fig. 1, e). As enlargement continued, several pyrenoids occasionally appeared, formed *de novo* and not by division of pre-existing pyrenoids. Often however no new pyrenoids were formed, the spore retaining its single pyrenoid throughout the period of enlargement. As development proceeded, the chloroplast became very dense, dark green and granular, obscuring the clear central zone and the pyrenoids, which however were still traceable under the immersion lens (Fig. 1, f).

The differential rate of enlargement within a single drop was very interesting. At first the crowded hypnospores formed an even curve marking the edge of the drop when it was first put on the agar. As the spores enlarged this curve was thrown into folds (Pl. XIII, A), the size of the folds depending on the density of the spores; when crowded together the spores remained comparatively small, while those at the edge of the mass or scattered singly enlarged more rapidly. In one such drop at the end of two weeks the hypnospores where crowded were small, some only 9μ in diameter, compressed and angular, whereas the more widely spaced spores were spherical and large, some as much as 37μ in diameter; between these extremes spores of all sizes occurred, depending on the degree of crowding (Pl. XIII, A, B).

By the end of December, after six weeks on agar, the agar was beginning to dry up and the colour of the spores had changed from green to gold once more and growth had apparently ceased. On the 5th January culture solution was added to one of the agar plates, submerging the whole surface.

Germination of the Spore. Development recommenced, the colour changed back again to green, and the larger spores began to germinate, the rest to continue enlarging. After two days there were many empty gaping spore-walls and scattered among them were large polyhedra. In this culture escape of the spore contents was not actually seen nor was

PLATE XIII. *Hydrodictyon patenaeforme*.

Asexual reproduction; living, unstained.

A—D from Groen Vlei, E—J from Grahamstown.

- A. Hypnospores developing round the edge of a drop of water on agar(861.) $\times 235$.
- B. Part of the same showing variation in size(862). $\times 520$.
- C. Hypnospores from the same plate showing parietal chloroplast, large pyrenoid, central vacuole and nucleus with large nucleolus(864). $\times 1100$.
- D. Older hypnospores from a culture made after the spores had nearly all died. Note two pyrenoids in some spores(1053). $\times 800$.
- E.—J. Development of young net. E, 3/5 life size. F—J, nearly life size.
- E. Young germ net of about 1,000 coenocytes.
- F. The same after a week in culture solution.
- G. The same five days later. H, Two days later than G, some of the coenocytes separating. J, One day later than H.

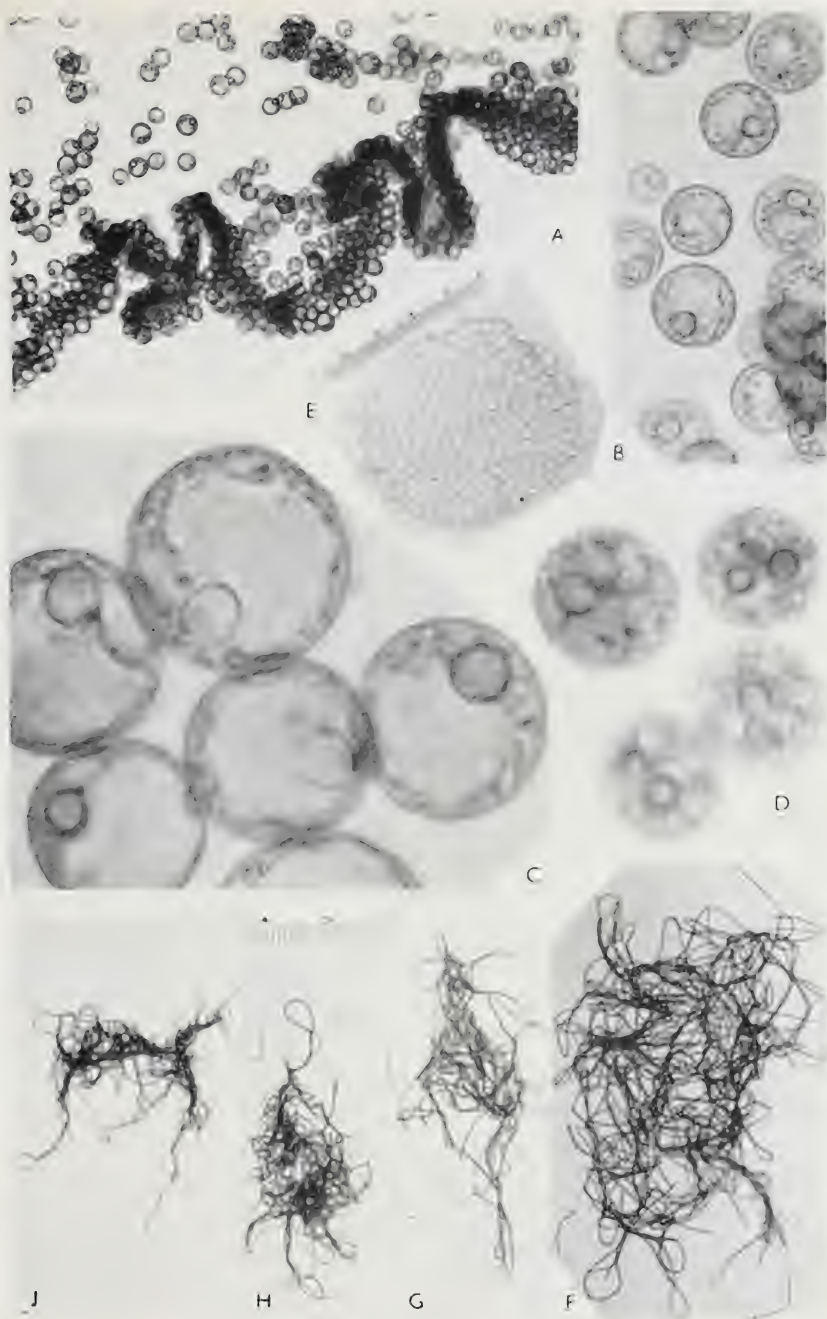


PLATE XIII. *Hydrodictyon patenaeforme*.

any motile stage observed; in the light of later experiments it was probably present but of brief duration. There was no sign of division in any of the spores, each apparently giving rise to a single polyhedron, diameter of spore-protoplast and newly formed polyhedron being approximately equal.

Development of the Polyhedron and Formation of the Germ Net. The resting spores which gave rise to polyhedra were all large (44μ — 58μ), hence the young polyhedron was also large and instead of having to undergo a prolonged period of growth, as appears to be usual in the field (cf. Pocock, p. 265), it soon attained its maximum size. Net formation quickly followed, just as in normal sexual reproduction. Even on the 7th January, only two days after addition of the culture solution, young germ nets were already present on the agar, while two days later many nets in varying stages of development were scattered over the plate.

Development of the Germ Net. Two or three germ nets were isolated, each placed separately in shallow dishes of culture solution. The nets enlarged rapidly, at first retaining their characteristic flat plate-like form (Pl. XIII, E), but soon, the individual coenocytes began to twist as they enlarged, the original shape was completely obscured and each net appeared as a tangled mass, becoming more tightly tangled as development continued (Pl. XIII, F—J). On the 16th February some of these nets, now dark green and densely tangled, were transferred to a jar of culture solution, packed in a hamper and taken by car to Grahamstown. Five days later the jar was removed from the hamper and left in a south window of the laboratory.

Swarms from the Germ Nets. The following day the water in the jar was green with swarms; the nets were breaking up, the majority of the coenocytes producing swarms just as in the previous generation. Again no conjugation was seen, but in iodine-fixed samples a few quadri-flagellate cells, obviously planozygotes, were found so that some cases of conjugation had certainly occurred. The swarms varied in size, some being distinctly smaller than others ($3 \times 4\mu$ and $4 \times 6\mu$).

Again drops containing swarms were put on agar and development proceeded as in the first case except that here, the nets being green, the swarms they produced and the resultant resting spores were also green from the beginning. Enlargement was followed by arrest of development with accompanying colour change to gold as the agar lost water.

Unfortunately, pressure of work prevented immediate germination experiments and when some months later attempts were made to obtain germination, drying up of the agar had proceeded so far as to injure the spores. These appeared healthy but on addition of culture solution they disintegrated. The chief value of these preliminary cultures lay in the

fact that they had shown the possibilities of the methods used for further detailed investigation of the life history of *Hydrodictyon*.

2. Material from Grahamstown.

During February and March 1938 nets were collected in a number of localities near Grahamstown and on several occasions swarming took place in the laboratory. For example, material brought into the laboratory on 5th March and put into water from the rain-water tank was found on the 7th to be liberating quantities of swarmers, green in colour, but otherwise both in structure and behaviour just like the Groen Vlei material. Here again no conjugation was seen, and put on agar, swarmers and spores developed similarly.

Evidently from these observations the formation of resting spores without conjugation is of common occurrence in *Hydrodictyon patenaeforme*. In all other respects such spores are similar both in structure and behaviour to those resulting from conjugation of gametes, i.e. zygospores, except that no division takes place on germination. The use of the term "parthenospore", suggesting as it does that the spore in question has arisen abnormally from a sexual unit which has failed to conjugate, is invidious since the formation of such spores appears to be a normal phenomenon in the life cycle of the species. The term "azygospore" is therefore used for all such spores whatever their origin. Though externally identical both in structure and development with the zygospore, the azygospore differs from it in genetical constitution, since the zygospore is diploid, the azygospore haploid. The nature of the swarmers concerned is discussed later.

B. CONFIRMATORY CULTURES.

To supplement and amplify the original observations many further experiments have been made with material from both the Cape Flats and Grahamstown. In some cases freshly collected nets, in others nets raised in culture were used. Of the latter, some were germ nets obtained from Miss Britten's material, others, nets raised in De Klip soil cultures, and yet others raised from spores derived from both sources and cultured on agar. Finally, plate cultures were made of motile cells found swarming in Groen Vlei itself.

During these experiments the whole life cycle—swarmer formation, development of hypnospores, germination, formation and development of polyhedra and finally germ-net formation—has been followed repeatedly, amplifying and to some extent modifying the original observations and resulting in a very complete picture of the life history of the species.

1. The Coenocyte.

As in *Hydrodictyon reticulatum* in the newly formed net of *H. patenaeforme* the chloroplast is in the form of a more or less complete, but somewhat irregular, peripheral girdle in the central region of each constituent cell. Enlargement is rapid partly owing to the development of a large central vacuole soon after the net-forming zooid has come to rest. Embedded in the chloroplast is a single large pyrenoid and lying within it adjacent to the vacuolar membrane is the single nucleus. As development proceeds the chloroplast becomes progressively more extensive, the degree to which it develops depending greatly on external conditions, while pyrenoids and nuclei multiply rapidly. The whole question of the structure of the coenocyte and more particularly of the chloroplast is dealt with fully later in the case of *H. reticulatum* and since all that is said there, with only slight modifications, applies equally well to *H. patenaeforme* it will suffice here to summarize the changes which take place preparatory to swarmer formation.

If conditions are favourable, that is, food supply ample, light sufficient and temperature reasonably warm, the chloroplast soon lines the entire wall as a homogeneous, translucent green sheet broken only by minute perforations always larger and more numerous towards the ends of the coenocyte; embedded in it are many pyrenoids of varying sizes while just within it lie numerous small nuclei (Pl. XIV, A, C). At this stage an organized chloroplast is no longer distinguishable—apparently the entire protoplast acts as one extensive fenestrated chloroplast.

This is the form at optimum development; if nutrition is poor or conditions otherwise unfavourable the coenocyte soon shows signs of starvation. The perforations become more extensive so that the cell is now lined by a much vacuolated chloroplast; through the vacuoles the nuclei may often be clearly seen (Pl. XIV, B), and the colour is paler, sometimes yellowish. In normal nets where growth is rapid the structure is usually intermediate between these two extremes, the perforations or vacuoles though present being small in proportion to the pigmented areas so that at low magnifications the chloroplast appears unbroken.

2. The Swarmers.

(a) Formation.

Left undisturbed, the coenocyte may remain in this condition for weeks, unchanged except for increase in size, but once maturity is reached swarmer formation may be induced by any sudden change in the surroundings. In culture, addition of fresh culture solution or transference to fresh water or culture solution may act as the trigger mechanism which

precipitates change of condition. The most certain and convenient method however was found to be transference to agar plates. Small portions of mature nets or isolated coenocytes put in drops of culture solution on agar plates nearly always produced swarmers the following day or in the course of a day or two, the time depending on the stage in development already reached by the coenocyte when it was put on the agar. Treated thus, development proceeds normally and individual coenocytes can be kept under observation.

The signs of approaching division are easily recognizable. It is even possible to pick out with the naked eye those coenocytes which are about to divide or are already in process of division. The colour first changes from the clear translucent green characteristic of the growing coenocyte to an intense dark green, then later changes again to an opaque rather yellowish green, the opacity being caused by the progressive breaking up of the chloroplast. This is specially marked when the coenocyte is viewed by reflected light and is a sure sign that division is nearing completion. Under the microscope, even at low magnifications, changes are apparent in both protoplast and wall, but for the accurate study of the finer details use of the 1/12 in. oil immersion lens on the living coenocyte is essential, despite the difficulty resulting from the large size of individual coenocytes.

With the beginning of preparations for division, the chloroplast loses its homogeneous appearance, the perforations increase in number and tend to coalesce, running in curved lines between which the green substance of the chloroplast begins to show intermittent thickenings, the whole assuming much the appearance of the whorls of a finger-print (Pl. XIV, D). Often many minute globules are present, while pyrenoids are still numerous. Macroscopically the colour-change to dark green now begins. At this stage again the coenocyte may remain quiescent for a considerable time or it may proceed at once to swarmer formation. The

PLATE XIV. *Hydrodictyon patenaeforme*.

Structure of the protoplast and preparation for division.

G from Grahamstown, the rest from Groen Vlei.

- A. Surface view showing pyrenoids of various sizes, fenestrated chloroplast with nuclei showing through the fenestrations(1070). $\times 800$.
- B. Another coenocyte, partially starved, with larger fenestrations or vacuoles in the chloroplast. Nuclei showing through some of the vacuoles(1066). $\times 1100$.
- C. Two pyrenoids in optical section(1069). $\times 1100$.
- D.—F. Preparation for division:—
- D. Showing "whorls" in the chloroplast, some comparatively small pyrenoids still present. (1065) $\times 800$.
- E. Division of chloroplast complete, granular stage(1068). $\times 800$.
- F. Cleavage beginning(1067). $\times 800$.
- G. Part of a coenocyte in which liberation of swarmers has begun. On the left, dark mass of swarmers, to the right swarmers separating from the mass, pushed into centre of the coenocyte by the contraction of the vacuolar membrane and expansion of the inner wall; laminations of the latter clearly apparent. (922) $\times 57$.

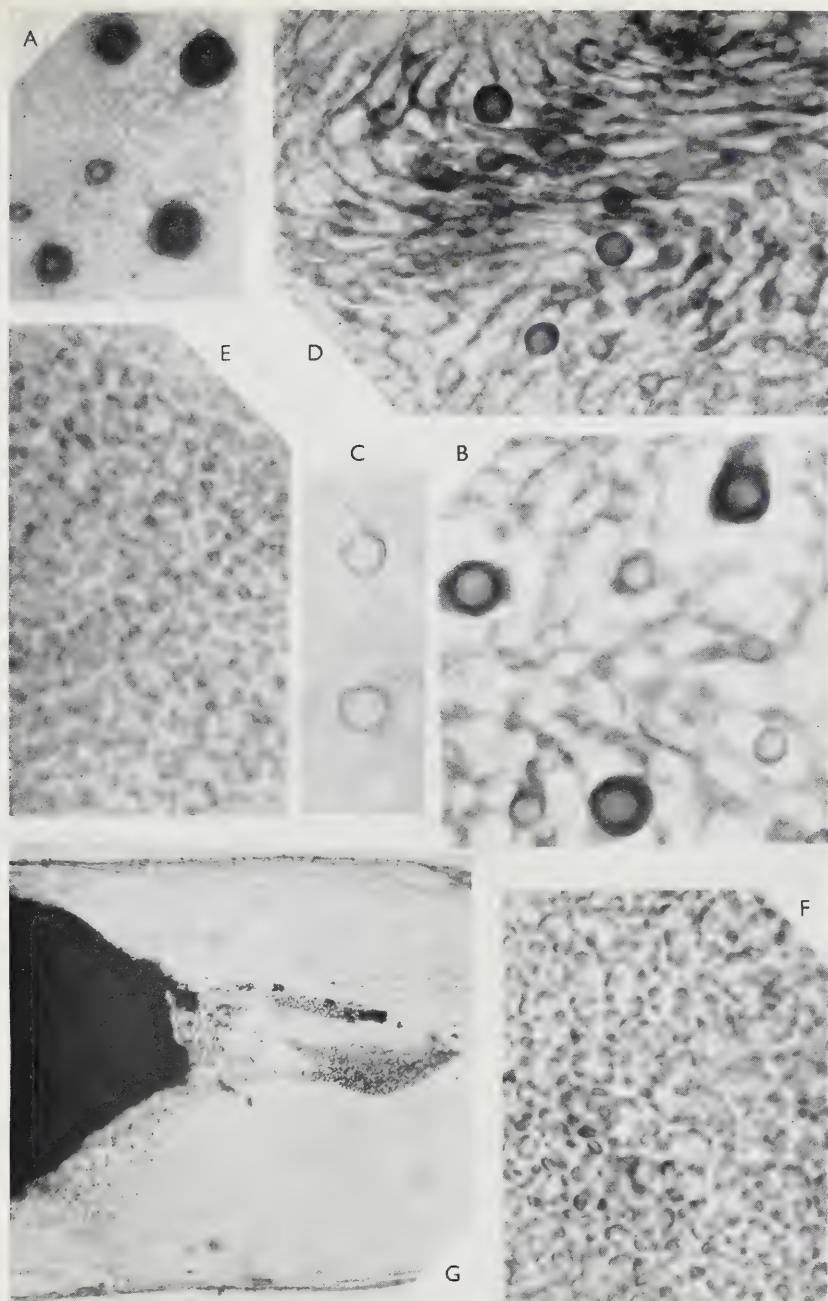


PLATE XIV. *Hydrodictyon patenaeforme*.

localized thickening separated by lines of perforations progresses until finally the whole chloroplast is divided up into innumerable small ovoid or spheroidal portions. Meanwhile the pyrenoids have been changing; first the outer angular starch sheath begins to dwindle, then the body of the pyrenoid appears corroded and often fragments; finally, the whole structure disappears so that when division of the chloroplast is complete usually no pyrenoids at all survive (Pl. XIV, E). The starch content of the cell, however, has not diminished; on the contrary, the dividing coenocyte is normally very rich in starch, but now, instead of being localized in or round the pyrenoids, it is disseminated in minute granules throughout the chloroplast, each small segment of which contains starch. This may appear as a thickening in the centre of each small plastid, visible even when unstained.

While these changes have been going on in the protoplast, changes in the wall have also been taking place. In the vegetative condition the wall appears as a thin tough membrane closely adpressed to the protoplast; now an inner hyaline mucilaginous zone can be seen separating the outer membrane from the protoplast—another feature which indicates preparation for division and which is easily distinguishable at low magnifications. As development proceeds this colourless zone of the wall widens, becoming more marked.

Meanwhile, repeated nuclear division has resulted in a very large number of small nuclei evenly distributed throughout the peripheral protoplast. Round each nucleus a number of small chloroplasts group themselves, sometimes associated with minute globules. Preparations for division of the protoplast are now complete and careful examination with the immersion lens reveals the following organization:—surface focus shows a continuous layer of granular chloroplasts; a slightly deeper focus shows the nuclei as colourless centres surrounded by a ring of plastids, while a still deeper focus again shows a continuous layer of plastids. Thus each nucleus is surrounded by a sheath of small chlorophyll-containing plastids. Seen macroscopically, the colour is now an opaque yellowish green, and again there may either be a period of arrested development before cleavage finally begins or the protoplast may proceed at once to the final stage in division. Cleavage is progressive; at first large masses are delimited, then these are cut up into smaller parts until finally the whole surface is seen to be divided into regular polygonal areas, most often but not always hexagonal and approximately $5-7\mu$ in diameter, the "pavement" stage described by Braun (p. 56) in *Hydrodictyon reticulatum* (Pl. XIV, E, F) and figured also by Oltmanns (I, p. 279, Fig. 187 (3)). A slight reorientation of the parts inside each small segment of the protoplast follows, a clear zone marking the anterior pole of the

swarmers forming to one side of each polygon. Division of the protoplast is now complete.

(b) *Liberation.*

Separation of the swarmers now begins; the space necessary for the process is obtained partly by a slight increase in size of the coenocyte and a decrease in size of the central vacuole, partly by changes in the swarmers themselves. These consist chiefly in a further reorientation of the component parts during which the diameter parallel to the surface decreases slightly while that perpendicular to the wall increases, but probably there is some loss of water from the cell sap of each swarmer as well as from the central vacuole of the coenocyte itself. In the early stages it is difficult to confirm the decrease in size of the latter owing to the density of the surface layers, but later in development it becomes very apparent.

Once movement has been initiated, it rapidly strengthens until the swarmers have all separated from one another and are moving independently in all directions. By this time the space between the outer wall and the central vacuole has noticeably increased, the inner layers of the wall swelling inwards to a considerable degree. In some cases the central vacuole, though obviously smaller, still persists and the swarmers continue to move in a peripheral layer. In other cases the vacuole contracts strongly and finally disappears altogether; when this happens the mass of swarmers contracts, forming a dense quivering column along the centre of the coenocyte from which individual swarmers gradually free themselves (Pl. XIV, G). In either case, the wall is finally ruptured, usually near the centre of the coenocyte, and the swarmers begin to escape. Normally, escape into the water is direct, but sometimes the swarmers hang together for a time in an ever enlarging mass, which eventually breaks asunder letting the swarmers disperse. Where this has been seen, as for instance in Miss Britten's material, the coenocytes had been kept for a time in a moist atmosphere and it is possible that they had lost a certain amount of water so that the cell sap had a higher density than usual; hence the swarmers were held in a slightly mucilaginous matrix until contact with the surrounding water had brought about the necessary adjustment. Nothing in the nature of a membrane enclosing a protruding vesicle has ever been observed in this species. Expulsion of the swarmers is aided by the swelling of the inner layers of the wall, the laminated structure of which becomes clearly visible (Pl. XIV, G) and it is probable that both the rupturing of the membrane and the swelling of the inner wall are caused by enzymes secreted by the active swarmers.

(c) *Number and Form.*

The number of swarmers formed is immense. When it is remembered that coenocytes over 2 cm. long and 1 mm. in diameter are not uncommon while at times they may be considerably larger, it is obvious that the number of swarmers formed per coenocyte must be far greater than in *H. reticulatum*. As against this, of course, the number of coenocytes in each net (usually about a thousand) is very much lower than in the latter species. A rough estimate of the number of swarmers produced by a single coenocyte may be obtained thus: regarding the protoplast as a cylinder and ignoring the closed ends, in the pavement stage the surface of the cylinder is completely divided into approximately equal polygonal areas, each $5-7\mu$ in diameter, which for the present purpose may be assumed to be hexagonal. If the area of the cylindrical surface be divided by that of a single hexagon, the quotient will give an approximation to the number of swarmers formed. Thus, an average-sized coenocyte 1.5 cm. long by 0.8 mm. in diameter (the thickness of the wall being negligible) which in the pavement stage is divided into segments 6μ in diameter, would produce over a million and a quarter (1,298,000 nearly) swarmers. In vigorous material all the coenocytes of a net isolated in culture solution may produce swarmers more or less simultaneously so that the water is coloured green while the "ghost" of the net is outlined by accumulations of swarmers along the empty walls of the component coenocytes.

In fully normal coenocytes division shows a high degree of regularity, but in adverse conditions, for example after a long time in culture or in badly contaminated cultures, division may be irregular, resulting in considerable variation in size of the segments and consequently of the swarmers. Again, normally segmentation takes place uniformly throughout the protoplast, but abnormal conditions may cause lagging, one part dividing before the rest, and instances have been seen in which, when swarming began in a coenocyte, part of the protoplast was still undivided and even degenerated without forming swarmers. In other cases part of the protoplast had been attacked by a fungus, yet the uninfected part formed apparently healthy swarmers.

The swarmers are minute, usually $3 \times 5\mu$ to $4-6\mu$, but sometimes larger, and are extremely active in their movements. There is some variation in their structure. Sometimes the several small chloroplasts coalesce to form a single oblique more or less lobed chloroplast which contains a dense portion rich in starch, probably of the nature of a pyrenoid (Fig. 1, a; cf. Pocock, p. 374). Such coalescence of chloroplasts occurs regularly at certain stages in the life cycle, namely in the hypno-spore as it develops and in the young polyhedron. Hence the appearance

of a single-lobed chloroplast in a swarmer itself simply means that the separate chloroplasts have coalesced rather earlier than usual. More usually several distinct chloroplasts each containing starch characterize the swarmer throughout its active life and persist into the early stage of the hypnospore. In such cases there is no trace of a pyrenoid (Fig. 1, b). The eyespot is as a rule conspicuous, rather narrow and elongated and placed slightly anterior to the equator or further back; occasionally it is poorly developed or even absent. The nucleus with large nucleolus lies in the clear anterior part near the apex of which a single contractile vacuole may sometimes be seen. The two flagella, about body length, are inserted separately at opposite sides of the apex.

The shape varies considerably according to the degree of activity; normally somewhat elongated with narrowly rounded ends, when very active the polar axis becomes longer in proportion to the equatorial and the poles are drawn out, the posterior pole at times becoming almost apiculate. This is most pronounced in cases where sexual attraction is strong, before actual association takes place. There is considerable plasticity, the normal form being resumed if the swarmer comes to rest, even momentarily.

(d) *Behaviour.*

The swarmers vary considerably in their behaviour. Most often, as in the first instance, they are strongly phototactic, but occasionally they show little or no response to light, scattering evenly over the drop of liquid when put on agar, and on coming to rest forming a rim all round the drop instead of congregating to one side; all degrees of sensitivity to light between these two extremes have been seen.

In the majority of the laboratory cultures made in 1939 and 1940, particularly early in the year, there was seldom any inclination to conjugate but from time to time material brought in from the field and later also some of the laboratory cultures, produced swarmers which conjugated actively. Coenocytes from such material put in a drop of water on agar completed their development and liberated swarmers which began to copulate immediately on liberation—small clumps formed as the swarmers escaped and the little clusters of some half-dozen or so swarmers scattered over the field, moving both as a whole and in respect to the individuals composing them, were most distinctive even under low power. In such cases the swarmers were uniformly small and extremely active, and the urge to conjugate so strong that any inclination to respond to light seemed to be masked.

Material collected early in the season 1941, in June and July, both from De Klip and Groen Vlei, behaved differently. Soon after liberation

the swarmers showed signs of "clumping" but were strongly phototactic and conjugation was often delayed; in some cases swarmers put on agar were still active 48 hours later. Among them were a few planozygotes but the majority had neither conjugated nor rounded off to form hypno-spores. Later experiments at Grahamstown, using nets collected locally, gave somewhat similar results—swarmers put on agar sometimes exhibited phototacty but more often showed no inclination to collect to one side of the drop. In the latter case in particular the majority disintegrated after some hours of movement and the few resting spores formed were scattered indiscriminately over the surface of the agar where the drop of water had lain. What factors bring about these differences in behaviour in still unknown.

Planozygotes may often be distinguished from gametes by their slightly larger size but as the swarmers themselves may vary in size, that is not sufficient criterion. Nor has it proved possible to discriminate among the swarmers except in so far as regards their behaviour; whether

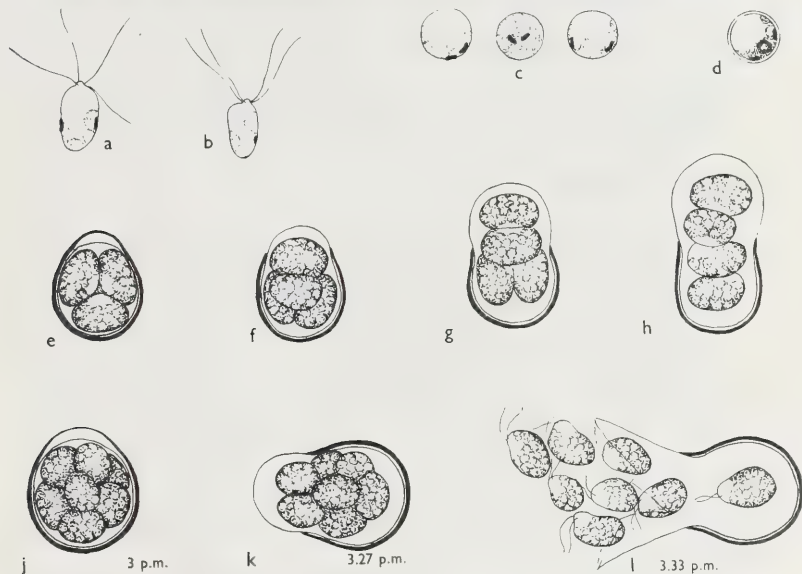


FIG. 2.—*H. patenaeforme*. Sexual Reproduction. a, b, planozygotes; c, young zygotes recently come to rest, two eyespots present; d, young zygospore, wall just formed, eyespots still present; e—h, germination of zygospore, four zoospores formed and about to escape; j—l, germination of larger zygospore, eight zoospores formed: j (3 p.m.), vesicle beginning to form; k (3.27 p.m.), vesicle enlarging, zoospores passing out into the vesicle and beginning to separate; l (3.33 p.m.), vesicle ruptured, zoospores escaping. a—d $\times 1000$; other figures $\times 500$.

some are gametes and others asexual in their nature or all potential gametes still remains to be determined. In iodine-fixed samples the planozygotes can at once be distinguished by the possession of four flagella and even when conjugation has not been general it is usually possible to find occasional quadriflagellate individuals, presumably planozygotes, here and there among the biflagellate swimmers. Examination of living material with an immersion lens reveals the presence of two eyespots both in planozygotes and the resting spores recently formed from them (Fig. 2, a, c, d).

(e) *Treatment.*

Whether liberated on agar or in liquid, swimmers were collected by means of capillary tubes or fine pipettes, collection being materially aided by the strong phototactism usually present, and transferred either to fresh culture solution in watch glasses, in which development may be watched both with low power and water-immersion lenses, or to drops of liquid culture solution on agar. The latter method was the one most used, although some of the watch-glass cultures were highly successful and served as a check on the agar method. Large numbers of cultures have been made at various times and the hypnospores thus obtained germinated. On the whole, the spores develop best on agar, but must usually be transferred to liquid before they can be induced to germinate.

3. The Hypnospore.

(a) *Formation.*

As the swimmers come to rest their form changes, becoming at first oval and then round, the clear apex gradually growing more broadly rounded, although the flagella may still be moving. At this stage the contractile vacuole (only one has been seen but possibly two may be present pulsating alternately) is most clearly distinguished. There is a strong tendency for the swimmers to collect on some solid surface, becoming attached by the tips of their flagella which are then gradually retracted until the body of the swimmer, now approximately spherical, is drawn into close contact with the supporting surface. Thus resting spores may accumulate over the walls of empty coenocytes, against soil particles, pieces of plant detritus, and also against one another, building up small masses of many spores.

As the flagella are withdrawn a delicate hyaline wall, which grows with the developing spore, is laid down; the eyespots are conspicuous and persist even after the wall has been differentiated but thereafter soon disappear. Consequently, while the hypnospores are young it is possible to distinguish azygotes with their single eyespot (Fig. 1, c) from zygo-spores in which two eyespots may be seen, either lying side by side or

more or less widely separated (Fig. 2, c, d). The eyespots are indeed curiously resistant. Often, particularly in hanging drops, swarmers at the edge of a drop disintegrate as the drop dries up or surface tension may cause the delicate limiting membrane to rupture, scattering the constituents of the protoplast into the water as an amorphous mass of rapidly disintegrating globules and granulea among which the orange-red eyespot is conspicuous, retaining its form and colour longer than any other part of the protoplast.

(b) Rate of Development.

The rate at which the hypnosporc develops depends directly on three external factors: food supply, water and temperature, to which a fourth, oxygen supply, should be added.

If newly formed spores are left in water poor in nutrient salts, they remain small and the colour soon changes from green to gold. Again, if the hypnospores are very closely crowded, as for instance when, left in liquid, the swarmers form a dense film of resting spores on the side of the container, the absorbing surface is reduced and a similar arrest of development with colour change from green to gold results even if the nutrient solution is replenished. If however the film is loosened and the spores separated from one another by gentle pressure under a cover slip and then put back into culture solution, the colour immediately begins to change back to green and growth is resumed.

On the other hand, in nature, close crowding of the spores must retard desiccation and the tendency of motile cells to crowd together in coming to rest and thus form large aggregations of hypnospores, is undoubtedly protective in its effects. The slightly mucilaginous outer surface of the wall keeps the hypnospores together and it is probably largely in the form of crowded clumps of minute golden spores that *Hydrodictyon* survives in this country since the early drying up of the ditches and vleis in which it occurs soon deprives the resting spores, unless formed early in the season, of both water and food.

The effect of desiccation in bringing about stoppage of growth accompanied by colour change was well seen in beaker cultures in which a thick film of spores had formed on the side of the beaker right up to the surface of the water. As the water evaporated the spores exposed turned golden while those in the water remained green, the golden zone extending gradually downwards as the water level fell. Similar results were seen in agar cultures in which the agar was beginning to dry up. In such cases when fresh water is added, even distilled water, many of the crowded spores recover, while those lying isolated on the agar are more liable to be injured by drying and soon degenerate.

Temperature plays a large part in determining the rate of growth of the spore. In the spring two agar plates inoculated at the same time with swarmers from a single culture both showed well formed hyphospores the day after inoculation. One was kept in a south window, the other in a warm room in a window with a northern aspect. In a few days the spores on the latter were large, the chloroplasts dense and granular and beginning to form haematochrome, whereas on the former in the colder room they were still small and green. Later in the season when room temperature was higher, the insolation in the north window was too strong, the spores tended to disintegrate, and the rate of development in the cooler room was the more rapid.

That the oxygen supply is also important was shown by some of the watch-glass cultures; while many of the swarmers settled down on the floor of the glass, others were held in the surface film of the culture fluid. The latter developed much more rapidly than those on the bottom of the watch glass, suggesting that development was hastened by superior oxygenation. This factor may also be a contributory cause to the good development of spores on agar plates.

(c) Germination: Methods and Results.

As in the initial experiments germination sometimes occurred on addition of culture solution to the agar plate, but more certain results were obtained by removing the spores from the agar and transferring them to liquid. At first normal culture solution was used but later it was found that better results were obtained by diluting the solution to half strength. If the spores to be germinated had been matured on agar, they were either picked off by means of capillary tubes or loosened with a soft brush (preferably sable), washed down to the edge of the plate, pipetted off, washed in several changes of glass-distilled water and finally transferred to 50 per cent culture solution in watch glasses. If raised in liquid, portions of the film of spores from the side of the culture vessel were placed on a slide, separated by gentle pressure and then treated similarly. The watch-glass cultures were watched by direct observation and also by placing samples from time to time on slides where more detailed observation was possible.

Germination often followed promptly, polyhedra being formed in less than 24 hours, but in other cases no change appeared for days. In such cases changing the culture medium sometimes induced germination. Transference to distilled water for some hours, then replacement of the water by culture solution was found effective in many cases. In others, particularly if the spores were old, the changes had to be repeated several

times before any sign of germination could be seen, while in a few cases none of the methods applied took effect. This reluctance to germinate was usually found in spores which, though apparently healthy, were old, large and thick walled and red-brown in colour.

The change in concentration of the surrounding liquid apparently acts as a "trigger" mechanism, to initiate the reactions which result in the necessary enzyme activity preliminary to germination. Exposure to direct sunlight for a brief period sometimes acted similarly, particularly early in the season, but in this alga direct sunlight is not essential to germination and in the shallow dishes used there was always danger that the temperature might be raised above the safe maximum.

The age of the spores and the state of development reached both play a part in determining the rate of germination. At first only large well-developed thick-walled spores with dense cell contents, already golden brown or beginning to turn brown, were used in the experiments, but later it was found that quite small spores, still bright green and with pyrenoid and central vacuole still conspicuous, germinated readily and more rapidly than larger older spores.* Further, in the various spore cultures it became apparent that the percentage of successful germinations was highest where the spores were young and green and still quite small, from 16μ upwards. In cultures of such spores many germinated in less than 24 hours while within two or three days nearly every spore had germinated. In older medium-sized spores the percentage of initial germinations was lower and spread over a longer period, after which several changes of medium were sometimes necessary before any of the remaining spores would germinate. Finally, in cultures of very large old thick-walled spores germination was usually delayed for a more or less extended period and often a number of the spores could not be induced to germinate at all, even by repeated changes of medium. On the whole, the younger the spore the more rapid was the rate of germination.

As against this, in some cases many of the zoospores formed in cultures of very young spores failed to form polyhedra and disintegrated, suggesting a higher degree of sensitivity to surrounding conditions than in the case of older spores; this was most marked where a very dilute culture solution (less than 50 per cent) had been used, so probably a different concentration of nutrient salts is needed. The fatty content of such young spores is of course far less than in older spores.

* Pringsheim (1861, p. 8) gives as the minimum size for germination in the spores of *H. reticulatum* $1/40$ mm., i.e. 25μ , but says it was more usually $1/30$ — $1/24$ mm., i.e. 33μ — 42μ .

Further, since the polyhedra formed from very young spores are themselves small, they must consequently undergo a longer period of enlargement before maturity is reached than those from larger spores and thus their chance of survival is correspondingly lessened. Alternatively, the germ net formed from the polyhedra must be smaller than usual.

In addition to the age factor a seasonal factor also appears to be operative. In cultures made in winter and spring the spores were usually induced to germinate comparatively easily; in those made during hot summer weather (in December and January), on the other hand, the spores were most reluctant to germinate. Since in the earlier cultures warmth had been found to assist in bringing about germination, this apparent seasonal difference may possibly be attributable rather to the smaller difference between day and night temperatures in summer than to the actual higher day temperature.

The possible results of germination show considerable variation. Observations of many germinations from different cultures show that spores fall into several categories according to their behaviour during germination, although it has not always been possible to determine in how far differences in behaviour result from inherent differences in the spores or from external influences. This applies particularly to the third class mentioned below, the first two classes including spores in which behaviour on germination may be regarded as normal, i.e. what might be expected in spores of different cytological equipment:—

Class 1. No division, nuclear or cytoplasmic, takes place. Each spore, no matter what its size, produces a single biflagellate, uninucleate zoospore (Fig. 1, g—k, r—s).

Class 2. Two successive nuclear divisions are followed by cytoplasmic cleavage into four equal parts, resulting in the formation of four uninucleate biflagellate zoospores (Fig. 2, e—h). If the spore is large a third division may take place resulting in eight instead of four zoospores (Fig. 2, j—l).

Class 3. Two nuclear divisions occur but cytoplasmic cleavage does not follow, or is transient, or incomplete. Here therefore there are several possibilities:—

- (a) No sign of division can be seen in the living spore (Fig. 3 g—j).
- (b) In the early stages of germination there is incipient cleavage of the cytoplasm, one or two lines of cleavage appearing at the side of the protoplast, only to disappear again as germination continues (Fig. 3, k).

In both these cases, as in Class 1, germination results in the formation of a single zoospore, but here, on closer inspection,

tion usually involving staining, e.g. with iodine, the zoospore is found to have four apical nuclei, each with its own flagellary apparatus.

- (c) The protoplast divides once only, either into two equal parts resulting in two binucleate, quadriflagellate zoospores, or into two unequal parts, in which case the smaller part forms a typical uninucleate biflagellate zoospore, the larger, one with three nuclei and three pairs of flagella (Fig. 3, a—c).
- (d) The protoplast divides twice, but the second division is incomplete and three zoospores are formed, two uninucleate, one larger binucleate and quadriflagellate (Fig. 3, d, e).

Class 4. As in the second variant of Class 2, three nuclear divisions occur, but cytoplasmic cleavage is incomplete, the third cleavage occurring in only some of the four parts resulting from the first cleavage, so that five, six or seven zoospores may result, varying in size but all uninucleate and biflagellate (Fig. 3, l, m).

It seems obvious that spores belonging to the first category are azygotes, the others zygotes. In the earlier cultures general conjugation of swarmers rarely occurred and in the young resting spore only one eyespot was as a rule present. Spores dividing during germination were conspicuously rare even when some cases of conjugation had been noted. In those of May—June 1941, on the other hand, conjugation was general and in nearly every case two eyespots were seen in the young spores examined, hence the assumption that the resulting hypnospores were zygotic seemed fully justified. Yet here again the majority of germinating spores formed one zoospore only and it was not until a closer examination revealed the presence of four nuclei in the apex and four pairs of flagella that the composite nature of the zoospore emerged. It must be emphasized that owing to the fineness of the flagella and density of the zoospore it is very difficult to determine the number of pairs of flagella except by staining, usually involving killing the zoospore; hence if the further development is to be watched, counting of the flagella is normally not possible, or at best very difficult, involving use of the immersion lens.

From these experiments then, it follows that, in *Hydrodictyon patenaeforme* at any rate, in distinguishing between zygosporos and azygosporos the presence or absence of division into four zoospores during germination is not sufficient criterion. Where division of the protoplast is absent so that a single zoospore is formed from each spore, the constitution of that zoospore must be considered. The presence of four nuclei, or four pairs of flagella in such a zoospore may be taken as sufficient indication of the zygotic nature of the spore from which it was formed.

What determines the behaviour of the protoplast in such cases is not

yet clear: all that can be said at present is that there seems to be inherent in this species a tendency to form one zoospore only instead of the four normally produced on germination of the zygospore in *H. reticulatum*.

Whatever its constitution, each zoospore forms a single polyhedron and hence gives rise to a single net.

(d) *Stages in Germination.*

The whole process of germination of both zygospores and azygospores has now been watched repeatedly so far as can be seen in the living state. No matter what the nature of the spore, the stages in germination follow the same pattern, except in so far as division is concerned. There is, however, considerable variation in detail, depending partly on the state of development previously reached by the individual spore, partly on external conditions.

If the spore is young, the chloroplast remains green and undivided and pyrenoids may be carried over into the zoospore (Fig. 3, g); this is however exceptional. Where it has been noticed the pyrenoids are usually beginning to disappear and usually disappear completely before the polyhedron is formed; there pyrenoids are formed *de novo*.

In older spores the usual sequence of changes culminating in germination is as follows:

1. The chloroplast loses its clear green translucence, becoming much darker in colour, congested with reserve food material, chiefly oil and starch; if germination is delayed, haematochrome is formed and the spore becomes golden brown in colour. During germination chlorophyll again appears, the haematochrome gradually disappearing, though in some cases traces may persist throughout the development of the polyhedron, surviving even into the germ net.

2. Lines appear in the chloroplast dividing it into irregular bands more or less radiating from the centre (Fig. 1, f); division continues until the whole chloroplast is divided into approximately equal parts, giving the spore a densely granular appearance (Fig. 1, h, j).

3. Meanwhile the pyrenoid or pyrenoids usually disappear completely, but occasionally may persist to a later stage. In the latter case, owing to the density of the chloroplast, pyrenoids even if present can only be distinguished with difficulty; often use of the immersion lens is needed to establish their presence.

4. The rich starch content is dispersed throughout the chloroplast, contributing to its congested appearance. The ratio of carbohydrate to other food reserves varies; in green spores it is high, in golden brown spores fatty food reserves predominate. The central clear zone is largely occluded, the chloroplast extending inwards towards the nucleus.

5. One part of the wall begins to thicken and project, the outer membrane stretching as a result of the swelling of the inner layers; below this projecting region the chloroplast retreats slightly (Fig. 1, g).

6. If the germinating spore is zygotic the first signs of division may appear at this stage (Fig. 2, e).

7. The swelling of the "polar region" increases until the outer membrane ruptures and the inner layers protrude (Fig. 1, h, j; Fig. 2, f), gradually swelling out to form the vesicle (Figs. 1, k, r, s; 2, g, h, k).

8. The protoplast begins to pass out of the spore, the vesicle gradually enlarging as the protoplast passes into it. If the spore is azygotic the whole protoplast slips out of the spore coat into the vesicle forming a single large zoospore (Fig. 1, k, r, s). If it is zygotic, either a single division precedes the passage of the spore contents into the vesicle where the second division is completed, or more usually the two divisions (three in the case of very large zygospores cf. Fig. 2, j, k) followed by cleavage of the cytoplasm are completed and the zoospores differentiated before the contents pass completely out of the spore coat (Fig. 2, f—h, k, l). In yet other cases incipient lines of cleavage appear only to disappear again either in part or completely before the zoospores are differentiated.

9. Differentiation of the zoospore or zoospores is completed, the flagella appear, and the zoospores where there are several, separate, flagellary activity beginning within the vesicle. In azygospores actual movement of the zoospore within the vesicle seldom occurs, but in germinating zygospores the zoospores usually become active before liberation.

10. The vesicle ruptures distally and the zoospores escape.

(e) Liberation of the Zoospores.

The mechanism of liberation may vary slightly in detail. The formation of the vesicle is controlled by the protoplast, the activity of which causes the inner wall to swell and brings about a marked increase in the osmotic pressure within the vesicle, both no doubt due to enzyme action on the food reserves of the spore. Internal pressure thus increases as the zoospores are differentiated forcing the inner membrane outwards until a large vesicle, bounded by a thin delicate membrane, is formed; the enlargement of the membrane keeps pace with the differentiation of the zoospores. This is best seen where there is only one zoospore which as it moves outwards, gets no nearer to the outer membrane from which it is separated by a clear zone of more or less constant width (Fig. 1, r, s). Eventually, as the pressure on the wall of the vesicle continues to increase, the limit of tensile strength is reached, the membrane ruptures at its distal end and the contents are forcibly expelled (Fig. 1, l). Thus in azygospores the single zoospore is shot or squeezed out through the

irregular orifice apparently remaining passive during expulsion; in zygo-spores sometimes the zoospores are expelled similarly as a single mass, or one or two are shot out, the rest following one after the other, either still passive or through their own activity. In the latter case they may be seen swimming about inside the remains of the vesicle before leisurely swimming out at the ruptured end (Fig. 2, l). Forceful expulsion is followed by a momentary quiescence on the part of the zoospore succeeded almost immediately by active movement. Once free, the zoospore swims vigorously but rather slowly and somewhat aimlessly, no eyespot being present.

(f) *Polarity of the Spore.*

In the resting spore there is no indication of polarity, but as soon as germination begins a definite polarity becomes apparent. There is no external evidence to show what determines the position of the region where thickening of the wall is to take place (cf. Fig. 1, h), but once it has started, the "apex" or anterior pole of the spore is fixed and its polarity is determined. Possibly, although not as yet observed, the nucleus moves from its central position to the surface of the protoplast, thus determining the anterior pole. In the zygospore, as the vesicle forms, the mass of the dividing protoplast follows more or less closely the shape of the expanding wall, so that there is a definite long axis. In the case of the azygospore the polarity of the protoplast is more marked and the long axis of the protoplast is also that of the single zoospore, although, since the slight asymmetry characteristic of all motile stages in this genus is more apparent in the zoospore than anywhere else, the apex of the zoospore is never at the actual pole but slightly to one side (Fig. 1, l, s).

A curious variation occurs in the azygospore. That end of the protoplast nearest the expanding "apex" of the spore would seem the obvious anterior pole of the zoospore and in many cases this is so, a clear region is visible slightly to one side of the apex and this becomes the anterior pole of the zoospore. From this region the flagella can be seen emerging, moving with the curious extending and retracting action peculiar to developing flagella and apparent at first merely as a flickering within the vesicle. When liberation takes place the zoospore consequently emerges with the anterior pole first (Fig. 1, k). In other cases, however, the polarity of the zoospore is inverted and the anterior pole develops at the inner end where the flagella begin to form as the zoospore slowly passes into the expanding vesicle. Hence in these cases when liberation takes place the zoospore is expelled backwards, and only after liberation does full flagellary action begin (Fig. 1, r, s). In those cultures in which this inverted polarity of the zoospore was noticed, it was exhibited by the

majority of the zoospores, whereas in other cultures normal polarity was the rule.

4. The Zoospore.

(a) Number and Size.

In germinating zygosporos as described above, if the spore is small or medium sized two divisions normally take place, yielding four zoospores; if however the spore has enlarged considerably prior to germination, the two initial divisions are followed by a third and eight zoospores result, approximately equal in size to the four formed from smaller spores. In those cases where unequal division takes place there is some variation in the size of the resultant zoospores. But, on the whole, except for the cases of unequal division enumerated in classes 3 and 4 above, the range in size of the zoospores produced as the result of either two or three divisions in germinating zygosporos lies within fairly narrow limits (from $10 \times 14\mu$ to $18 \times 23\mu$). It follows therefore that the number formed from any zoospore will depend directly on the size of the germinating zygosporos.

In azygosporos, on the other hand, since only one zoospore is formed no matter what the size of the spore and since the latter may vary from 16μ to over 50μ , it follows that the range in size of azygotically formed zoospores is enormous and the polyhedra to which they give rise correspondingly varied in size. The larger polyhedra undergo a much shorter period of enlargement subsequent to their formation.

With regard to the number of zoospores formed on germination, the present series of experiments amply confirm Pringsheim's observations in the case of *H. reticulatum*: whereas the number in azygotes is invariably one, and the normal numbers in the case of zygosporos are four or eight, in the latter any number from one to eight may on occasion be formed.

(b) Abnormalities.

The various cases of abnormalities due to irregularities in division of the zygosporos have already been noted, but one such case in which development was watched may serve as an illustration: The spore in question when first observed (9.10 p.m.) had nearly completed germination. Three zoospores, two of normal size ($21 \times 30\mu$ and $26 \times 30\mu$) and one about twice the size ($30 \times 40\mu$), had nearly passed out of the spore-coat into the vesicle (Fig. 3, d); ten minutes later the vesicle ruptured and the zoospores passed out into the water—the smaller ones first—where they swam about for a short time (Fig. 3, e). After a few minutes of activity, movement began to slow down and soon ceased; as the zoospores came

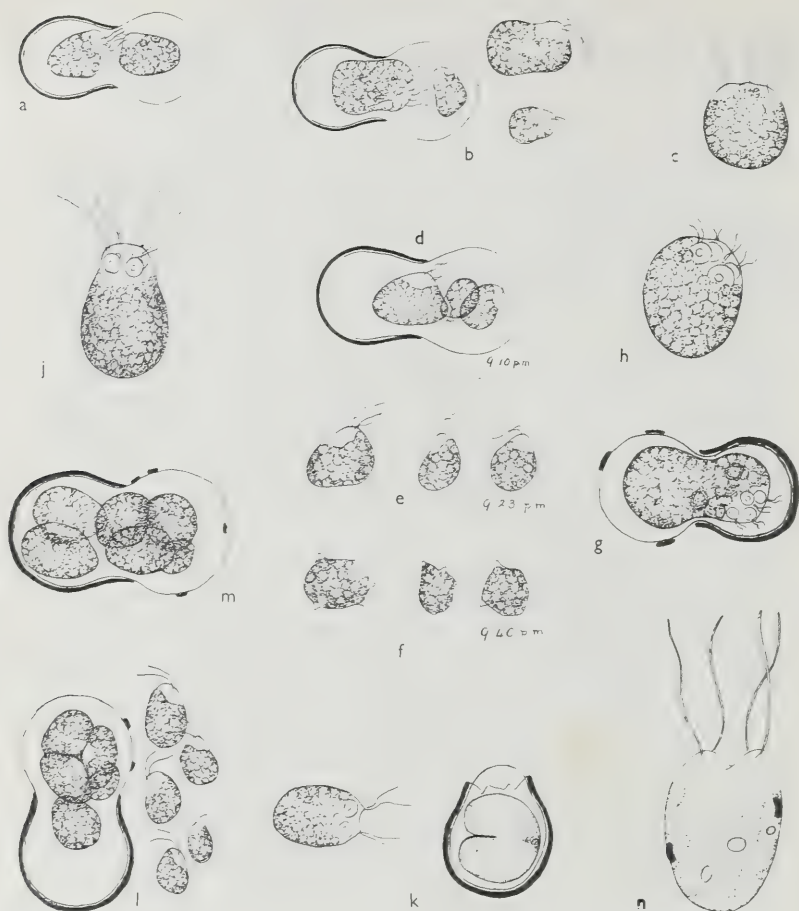


FIG. 3.—*H. patenaeforme*. Sexual Reproduction, variations from the normal. a, two equal zoospores formed, each with two pairs of flagella; b, two zoospores formed, one normal, the other three times the size with three pairs of flagella, shown inside the vesicle and after escape; c, zoospore with three pairs of flagella; d—f, three zoospores formed, one double the size of the other two, with two pairs of flagella: d (9.10 p.m.), just about to escape; e (9.23 p.m.), zoospores free, active, and f (9.40 p.m.), forming polyhedra; g, nuclear division complete, cleavage absent, one zoospore with four nuclei, four pairs of flagella forming, about to emerge backwards, traces of pyrenoids present; h, the same zoospore after escape; j, another quadrinucleate zoospore; k, partial cleavage resulting in two biflagellate, binucleate zoospores; l, five zoospores formed, shown passing into the vesicle not yet completely separated, and after escape; m, six zoospores of various sizes; n, zygote showing abnormal fusion.

n $\times 2000$; other figures all $\times 500$.

to rest the flagella were withdrawn and protoplasmic processes were thrown out which rapidly formed blunt conical processes and by 9.40 p.m. the three polyhedra were already formed although the processes were still short and blunt, their tips already colourless; in the largest spore the colourless apex was still distinguishable. Escape and polyhedron formation in this case were rapid though not abnormally so. Of the three polyhedra, the two smaller were undoubtedly uninucleate, the third almost as certainly binucleate, but since their development was being watched, this could not be verified by staining.

Sometimes a compound zoospore may result from fusion between two already differentiated zoospores; in such cases the resultant zoospore may be "two headed", i.e., with two separate flagellated apices. When this is the case, movement is usually slower than in normal zoospores and the separate pairs of flagella are more easily seen even in life.

Such compound zoospores may represent two, three or four normal zoospores, and treatment with iodine at once reveals their nature by the number of nuclei in the apical region and the corresponding number of pairs of flagella. But whatever their constitution, each forms a single polyhedron, somewhat larger than the average and from the first containing more than one nucleus. Occasionally if the zoospores have been only partially united, the resultant polyhedron may be more or less lobed but in this species such compound polyhedra rarely occurred.

(c) *Form and Behaviour.*

In germinating zygospores, where division during germination has been complete and pyrenoids have disappeared, the resultant zoospores are like those figured by Pringsheim (1861, Fig. 9, etc.) for *H. reticulatum*, though usually slightly smaller than the dimensions given by him for that species ($10 \times 14\mu$ to $18 \times 23\mu$ as against $21 \times 26\mu$ to $22 \times 33\mu$ estimated by Pringsheim). If the germinating spores were well developed and still green, the zoospores are an intense dark green, very granular in appearance. Under the high power the granules are seen to be distinct chlorophyll containing bodies (Fig. 2, l). In other cases where the germinating spore was very young and the pyrenoid persistent, the chloroplast of the zoospore was also bright green, rather granular in appearance but apparently not divided and there might even be a pyrenoid.

At the other extreme, in large brown spores the mass of fatty reserve and the haematochrome obscure the structure of the zoospore and the chloroplast, although very granular, is apparently not divided; probably, however, this appearance is due merely to the density of the zoospore which makes it impossible to distinguish divisions in the chloroplast. Such zoospores are deep golden brown in colour as are the polyhedra

formed by them, the chloroplast only resuming its clear green colour as the polyhedron develops; occasionally some haematochrome may persist even in the polyhedron. Zoospores of this type are more characteristic of azygospore germination and are often very large. As a consequence the resultant polyhedra mature in a very short time before the fatty reserves are used up and oily globules coloured reddish gold with haematochrome may still persist when the germ net is formed, lying among the meshes of the net, not inside the constituent cells.

The structure of the chloroplast is perhaps best seen in the zoospore formed by a rather large azygospore just before it escapes. It can then be examined with the immersion lens and the distinct parts into which the chloroplast has been divided clearly distinguished (Fig. 1, k, r, s).

The zoospore is typically slightly asymmetrical, the clear apex in which one or two moving granules may sometimes be seen, lying slightly to one side of the anterior pole; the flagella are inserted separately, rather wide apart, tend to be directed backwards, and since they are comparatively short, less than body length and very fine in proportion to the dense zoospore, are usually difficult to see in life and even sometimes when fixed. There is no eyespot and no contractile vacuoles have been seen (Figs. 1, k, s; 2, l and 3, e, l).

(d) *Movement.*

If the zoospore is small, movement after the momentary pause following liberation is normally energetic and continuous though not very rapid; sometimes, particularly in large zoospores, movement is slow and sluggish. There is no indication of any response to light. Sometimes the zoospores move up to the surface of the water, more often they move somewhat aimlessly over the floor of the culture vessel among the ungerminated or germinating spores and already formed polyhedra, showing considerable metabolic activity (Pl. XV. B), but the changes in shape never become amoeboid in nature; they more closely resemble the slight changes shown by many species of *Euglena* while still motile.

PLATE XV. *Hydrodictyon patenaeforme*.

C—E, Kimberley culture; the rest, Grahamstown.

- A. Hypnospores, one beginning to germinate, and one polyhedron. (962) $\times$ 375.
- B. Group of hypnospores, some germinating, zoospores and polyhedra, newly formed, and older, well developed, showing parietal chloroplast and large central vacuole(960). $\times$ 140.
- C. Young net (503 coenocytes)(717). $\times$ 240.
- D. Slightly older net (508 coenocytes), cells cylindrical(712). $\times$ 180.
- E. Cells from older net showing marginal processes (stained iodine). $\times$ 800.
- F.—J. Abnormal coenocytes: F. "Bubbly" coenocytes formed direct from polyhedra. (1000) $\times$ 17.5.
- G. Hypnospore and coenocyte direct from swarmers(958). $\times$ 150.
- H. Coenocyte formed from a germ-net zooid(959). $\times$ 235.
- J. Coenocyte formed direct from a hypnospore, above, a hypnosporea, below, polyhedron(963). $\times$ 140.

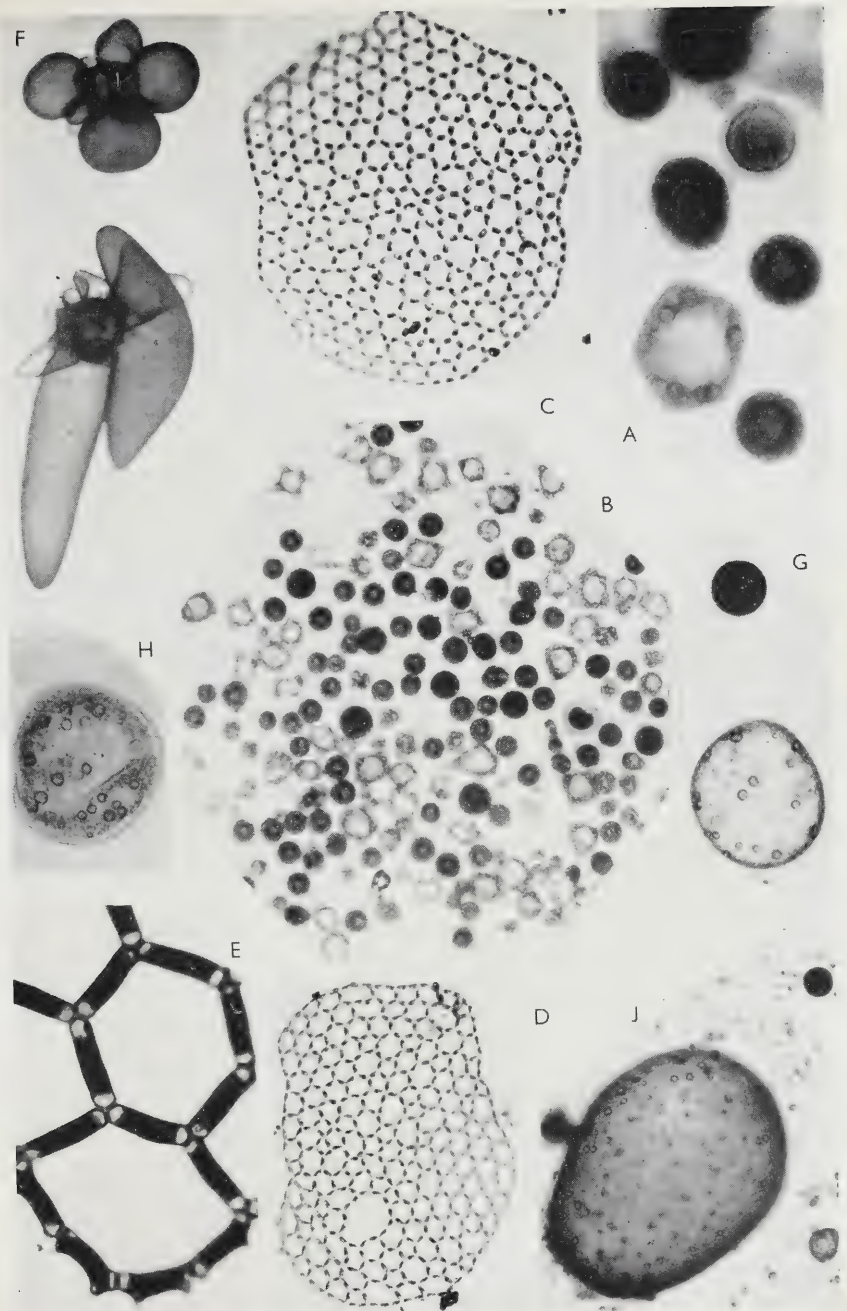


PLATE XV. *Hydrodictyon patenaeforme*.

Movement is never long continued; it may last only a few minutes or as long as half an hour or more. In a dozen cases which were timed from the moment of escape up to cessation of movement the time varied from 6—40 minutes, the majority varying from 15—30 minutes. Sometimes the motile period is even shorter, or may be omitted altogether. In the latter case, flagella are not developed, the contents of the germinating spore forming polyhedra directly either on liberation or inside the vesicle (Fig. 1, q). Probably one hour would represent the extreme limit of motility, but it is normally of very much shorter duration.

Since the motile period of the zoospore, even at its longest, is so brief it is not surprising that previous observations of this species in the field failed to establish its existence.

5. The Polyhedron.

(a) Formation.

Cessation of the movement of the zoospore is usually abrupt and accompanied by changes, notably a change in shape accompanied by a slight decrease in size. Sometimes delicate colourless thread-like processes are put out, as described by Mainx (p. 513) in *H. reticulatum*. These serve to anchor the zoospore and often one or both of the flagella may act similarly (Fig. 1, n), the apex becoming attached to the surface of the substratum. The flagellum then contracts, thickening as it does so, but its identity is soon obscured and the flagellum can no longer be distinguished from the other processes. These may show some movement as well as alteration in shape; they may even be withdrawn and others put out as the development of the polyhedron continues. Eventually conical projections are formed on the surface of the polyhedron, each originating in one of the processes, and a delicate wall is secreted over the whole structure.

At first thin throughout, the wall gradually thickens, particularly over the apices of the conical projections where the inner layers accumulate, so that eventually a thick hyaline spine-like structure results (Fig. 1, k—p).

In other cases the polyhedron forms more rapidly; without the emission of protoplasmic processes, conical projections appear at several spots on the surface of the quiescent zoospore, rapidly increasing in size; this was the case in the germinating spore described above (Fig. 3, c—f) in which the polyhedra were already formed within 20 minutes of the escape of the zoospores. In another case the polyhedra formed in less than 10 minutes.

(b) Development.

The protoplast of the polyhedron at first extends some way into the hollow projections which often contain moving granules; later as the wall thickens the apical regions of the projections become solid while the protoplast retreats more or less completely from the basal parts, consequently becoming approximately spherical.

The protoplast now passes through the same series of changes, but in the reverse order, as that which led to the formation of the zoospore, and similar to those seen in the developing coenocyte and hypnospore. The distinct chloroplasts coalesce to form a continuous pigmented layer: at first somewhat granular, the granules soon disappear, pyrenoids are formed, nuclear division progresses, a central vacuole forms rapidly increasing in size—in short, the polyhedron undergoes all the changes which in this genus characterize every stage of active vegetative growth. Ultimately the polyhedron shows a clear translucent green peripheral chloroplast, minutely fenestrated, containing many pyrenoids and just within it many nuclei surrounding the large central vacuole. Normally it is more or less isodiametric with projecting conical processes distributed over its surface, in fact a typical "polyhedron", often remarkably regular in shape. This external form is the sole idiosyncrasy which distinguishes it from all other stages of active growth, although there are slight differences in detail: it differs from the young coenocyte in that the chloroplast is more massive in proportion to the central vacuole, the wall thicker owing to the greater development of the inner hyaline layers, particularly in the region of the angular projections and in general the earlier commencement of nuclear division.

The changes in the polyhedron often take place very rapidly and may be completed within a few hours. The time needed to reach maturity depends partly on the size of the newly formed polyhedron and therefore on that of the zoospore from which it was formed, partly on external factors, chiefly food supply and temperature.

Just as in the coenocyte so in the polyhedron, during development the chloroplast may become more or less extensively fenestrated; when this occurs, the nuclei, usually difficult to distinguish, may be comparatively easily seen. Examination of the surface of such a polyhedron with the immersion lens reveals numerous nuclei through the fenestrations; usually one nucleus can be seen lying almost beneath each of the larger fenestrations. An optical section shows the nuclei arranged peripherally partly embedded in the inner layer of the chloroplast, partly projecting from it like the pyrenoids, but owing to their smaller size to a much less extent. When stained with iodine the nucleolus shows clearly. When

there is superabundance of food material the chloroplast is denser, the fenestrations smaller or absent and as a consequence the internal structure is obscured by the density of the outer layers.

6. The Germ Net.

The process of germ-net formation in *H. patenaeforme* has already been described (Pocock, p. 267, *et seq.*), but as a result of many further observations the account there given must be slightly modified in one or two particulars and amplified in others.

(a) Changes in the Protoplast.

A similar series of changes to that seen in a coenocyte which is preparing to divide, but necessarily on a much smaller scale, can be followed in the polyhedron as it prepares for germ-net formation. The chloroplast becomes densely granular and ultimately completely divided into small distinct chloroplasts, the pyrenoids disappear, the starch content being disseminated throughout the chloroplasts, while nuclear division proceeds actively. Enzymes secreted by the protoplast act on the substances composing the wall, particularly the inner layers, and when the polyhedron is ready for net formation, it appears as a dense green granular body surrounded by a wall in which the thin outer membrane is separated from the protoplast by a colourless hyaline layer, narrow except in the conical spines.

(b) Fate of the Polyhedral Wall.

Before net formation can begin a vesicle must be formed in which the process can be safely completed, this vesicle providing ample room for the several stages of formation of the zooids, their rearrangement and reunion, and at the same time protection for the young net.

In the cases previously described where polyhedra were brought in from the field and their subsequent development watched, the material of the wall was usually wholly used in the formation of the vesicle. But this is by no means always the case—more often the inner layers only are utilized in the process of vesicle formation, the outer membrane being sloughed off as the inner layers expand much as described by Pringsheim for *H. reticulatum*. Apparently the state of the wall depends partly on the age of the polyhedron and partly on external conditions. If the polyhedron has developed rapidly in freshly formed pools the whole wall remains relatively soft, whereas in less favourable conditions the outer membrane is altered to form a more resistant skin which does not gelatinize but is thrown off when the inner layers expand to form the vesicle. This skin is very delicate and thin even over the spine-like pro-

cesses, the form of which it may retain, so that it appears as a delicate crumpled ghost of the polyhedron on one side of the vesicle, to which it often adheres (Fig. 4, a). Most of the hyaline substance of the spines is made up of the same substance as the inner layer of the polyhedron wall and thus contributes to the wall of the vesicle.

(c) *Persistence of the Central Vacuole.*

As the preparatory changes take place in the protoplast, the sharp distinction between the parietal green layers and the central vacuole is obscured and the latter appears to be more or less obliterated. In normal net formation however this is not the case. On the contrary, the central vacuole, hidden by the densely granular chloroplast, persists and plays an important part in the early stages of net formation. As movement of the zooids begins, the inner layers of the wall swell and the vesicle begins to form. At first small, the vacuolar membrane is sufficiently large to keep the zooids in a peripheral position between it and the vesicle wall, this preventing the mass of agitated cells from collapsing on itself

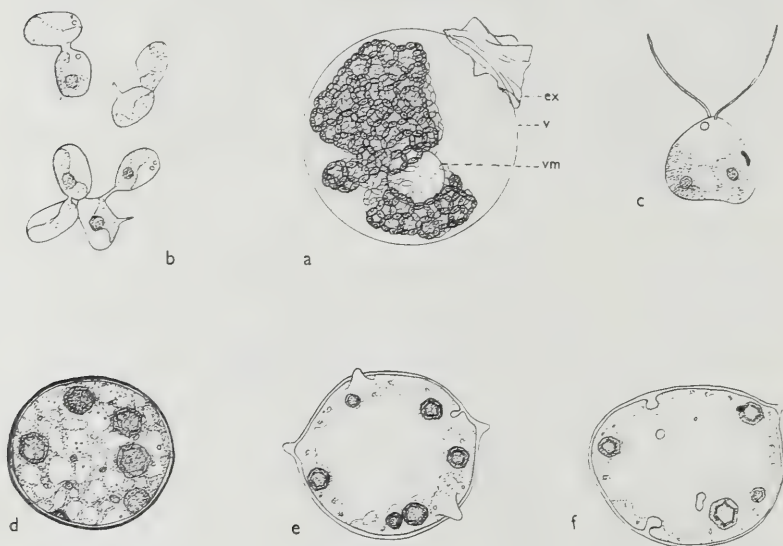


FIG. 4.—*H. patenaeforme*. Formation of the germ net. a, germ net just formed, vacuolar membrane (vm) persistent, net an irregular sac, outer membrane of polyhedron sloughed off (ex), adhering to the vesicle wall (v); b, abnormal fusion of zooids in net formation showing connecting strands; c, exceptionally large swarmer; d—f, coenocytes formed direct, d from a planozygote, and e, f, from zoospores, showing some indication of polyhedral spines and "pegs" of wall substance protruding inwards from the wall. a $\times 125$; c $\times 1500$; other figures $\times 500$.

or uniting in haphazard fashion across the centre. As the vesicle continues to enlarge the zooids tend to move outwards with it, the space between it and the vacuole increases and eventually, the function of the vacuolar membrane achieved, all relation between the two membranes is lost. Nevertheless, the vacuole enclosed in its membrane may sometimes persist, even after the net is completely formed. In one such case (Fig. 4, a) when first seen the net was already formed, but since some of the zooids still showed pulsating vacuoles, cessation of movement must have been very recent. The culture was an old one and many nets, including the one under consideration, were incompletely flattened. This particular net was irregularly curved to form an open hollow structure within which could be seen the central vacuole of the polyhedron enclosed in a delicate hyaline membrane. Dilute methylene blue was run in; the matrix surrounding the net was slightly mucilaginous staining faintly blue. The wall of the vesicle, much attenuated, stained slightly darker blue, while the crumpled outer skin, stained dark blue, still adhered to one side of it. The contents of the vesicle were light blue-purple, the young net more deeply stained, while the vacuolar membrane and its contents remained unstained and consequently very conspicuous for a few seconds; then the membrane collapsed and the space it had enclosed assumed the same colour as the rest of the vesicle.

Often however the central vacuole cannot be seen and it is probable that in most cases its work in early stages of net formation completed, it disappears completely before the net is fully formed; it is always a very delicate structure. Its importance will be more apparent when daughter-net formation in *H. reticulatum* is considered. In *H. patenaeforme*, since the germ net is typically a flat plate, the function of the vacuolar membrane in its formation must obviously end with the flattening out of the mass of zooids.

(d) Union of the Zooids.

In *H. patenaeforme* in nature and in clean vigorous cultures the germ net is almost always formed entirely in one plane, double-centred nets forming a very small proportion of the total number formed, but in contaminated cultures the mass of zooids often fails to flatten out, separation being incomplete so that the resultant net may be a tangled mass, the zooids uniting at random in several planes. In such cases it is probable that the central vacuole has broken down at a very early stage. On the other hand, in some cultures separation of the zooids proceeded further than usual and the resultant nets were not only flat but showed a large gap in the middle.

The actual mechanism of reunion among the zooids is the same as

in the daughter nets of *H. reticulatum* and will be considered later. Union is effected by protoplasmic processes put out by the zooids; usually these are so short as to be practically indistinguishable, but occasionally irregularities in net formation may cause the zooids to be slightly separated from one another and the connecting strands can then be seen. This occurred in several parts of the germ net described above (Fig. 4, b).

(e) *Time Required to Obtain Germ Nets from Swarmers.*

A number of experiments have been made to ascertain how long it would take to obtain new nets from swarmers. In these hypnospores were developed in optimum conditions and when they had reached a fair size they were transferred to 50 per cent culture solution to germinate. If the polyhedron is large when first formed development is often very rapid since the period of enlargement is short, and the germ net may be formed within 48 hours from the time of germination. It follows that, in general, other conditions being equal, zygosporcs will take longer to complete germination, polyhedron and net formation than azygosporcs of the same size. To begin with, there is an initial delay due to the necessity for reduction division in the zygosporc and, secondly, the zoosporcs and the polyhedra they form are smaller and consequently the latter has to undergo a longer period of enlargement before it is ready to form the germ net. As a rule the larger the spore at germination the sooner will maturity be attained by the resulting polyhedron. Hence the time which must elapse between germination of the spore and net formation is shortest in the case of very large azygosporcs. On the other hand, where the spore itself is large the time between the formation of the hypnosporc by a swarmer and germination will in general be longer than in the case of a smaller spore.

Taking all these factors into consideration, by careful selection of material and adjustment of conditions so that at each stage they were the best obtainable, it was found possible to shorten the requisite period considerably and in many cases germ nets of both *H. patenaeforme* and *H. reticulatum* were obtained within a fortnight of swarmer formation, while in a few cultures germ nets were formed by the thirteenth day, i.e. swarmers were liberated, formed hypnospores which matured and germinated, zoosporcs were liberated and formed polyhedra which in turn produced germ nets, all within a space of 12½ days.

From this it is obvious that, while hypnospores may undoubtedly be long lived, a prolonged period of rest is not essential and development may be more or less continuous, but it seems probable that this (i.e. 12½ days) is the minimum period within which the various stages can be completed. In such cases where the vegetative phases are reduced to

the minimum, several cycles of development may be completed in the course of a single season.

In some cases hypnospores and polyhedra may continue to enlarge until far bigger than usual and may remain quiescent after reaching their maximum size. In such cases both spore and polyhedron often become congested and very dark in colour. Repeated observations in very many different cultures make it evident that in all such cases again environmental conditions play an important role in determining the rate of development.

7. Deviations from the Normal Development.

The most striking deviations from the normal course of development were always connected with the behaviour of the motile cell.

(a) A motile cell occurring at any stage, whether a swarmer liberated from a coenocyte, a net-forming zooid, a planozygote or a zoospore produced on germination of a hypnospore, may on occasion develop directly into a coenocyte. Such a coenocyte develops just as if it formed part of a net, except that, being isolated it often tends to be isodiametric in shape. Those formed from swarmers (Pl. XV, G), or planozygotes (Fig. 4, d) or germ-net zooids (Pl. XV, H) are typically more or less globose with smooth walls, but occasionally a single such coenocyte puts out branches and the whole structure may become complicated like a series of soap bubbles (Pl. XV, F). Finally, coenocytes formed direct from zoospores often develop conical outgrowths showing despite their size their affinity with normal polyhedra (Fig. 4, e, f). But no matter what their origin, in this species such solitary coenocytes ultimately produce swarmers, never nets.

(b) Failure to develop flagella may result in the zoospores formed from germinating spores passing at once into the polyhedral stage either within or just outside the vesicle (Fig. 1, q). This elision of the motile stage is probably due to adverse conditions.

(c) Incomplete septation when the contents of a coenocyte or germinating zygospor divide may result in curious monstrosities—compound swarmers with one, two (Fig. 3, n) or more pairs of flagella in the former case, compound zoospores in the latter (Fig. 3, c, h, j). The polyhedra formed by such zoospores may be peculiar in shape but develop normally.

All these abnormalities have been noticed repeatedly in cultures. To what extent they may occur in nature is unknown, but it seems probable that they are in large part due to cultural conditions. In germination experiments abnormalities were more frequent in cultures of old spores; where young, recently formed hypnospores were used development

tended to be very uniform. This was perhaps more noticeable in germ-net formation than at any other stage; tangled or otherwise abnormal germ nets were common in old, crowded, or contaminated cultures, whereas in nature or in healthy cultures of young spores perfect plate-shaped nets composed of coenocytes united throughout in one plane only were almost invariably formed from the polyhedra.

8. EXAMPLES OF SPORE CULTURES.

To exemplify the variations in different cultures the following cases have been selected:

(i) Coenocytes from a net hatched in a culture of soil from De Klip on 18th January 1940 were put on agar on the 24th February. On 3rd March swimmers were liberated and transferred to a watch glass of culture solution where they settled down forming a dense film on the floor of the glass. A fortnight later (18th March) the liquid was found to have evaporated, but on addition of fresh culture solution the spores were found to be uninjured, while a dense felt of fungal hyphae growing among them had been killed by the drying. The spores remained bright green and comparatively small, ranging up to 35μ but many being much smaller. Nearly all contained a single pyrenoid, only an occasional one having two. The culture was kept and from time to time parts of the layer of spores were used for germination experiments. On 5th June part of the spore layer was removed, washed with distilled water on a slide and the spores separated by gentle pressure under a coverslip. After further washing the loosened spores were transferred by means of capillary tubes to a watch glass of fresh culture solution made with soil decoction from De Klip soil.

Twenty-four hours later there were already many polyhedra present as well as a number of still motile zoospores, while most of the remaining spores showed various stages in germination. By the following evening there were only a few isolated groups of small spores still ungerminated. The germinating spores ranged in size from 16μ to 35μ , the majority being about $22-26\mu$.

In this watch-glass culture the pyrenoids had not multiplied, each spore, with few exceptions, retaining a single pyrenoid throughout its development until the final disappearance of pyrenoids in the last stages leading to germination. The spores were not large, the chloroplast intensely green throughout becoming densely granular before germination; examination with the immersion lens showed it to be divided into a mass of small rounded portions. The spores were all azygotic, and each formed a single zoospore; among the many germinations watched not one showed any sign of division.

Further, in all the cases seen, this culture showed the inverse polarity described above, i.e. in all the flagella formed at the inner pole and the zoospore escaped backwards (Fig. 1, r, s). Before escape flagellary activity could be clearly seen, the flagella showing characteristic undulatory movements in the empty space left in the spore as the zoospore slowly slid backwards through the narrow opening into the vesicle. In the vesicle the movement of the flagella strengthened without however imparting movement to the zoospore; this remained passive during extrusion into the vesicle and subsequent rupture of the latter which nearly always followed without a minute's delay. Another feature of this culture was the vigorous, comparatively rapid movement of the liberated zoospores, which also showed marked metabolic activity, changing shape as they moved. Both types of movement were particularly strong and ended abruptly. The polyhedra formed rapidly, conical projections beginning to appear as soon as movement ceased, often without previous emission of hyaline processes.

Throughout the culture, each stage showed a high standard of vigour and normality and considerable uniformity in the spores. Since the spores themselves were small, the resulting polyhedra were also small and therefore proportionately slow in reaching maturity.

In this culture maturation of the spores had taken place in liquid, interrupted by a brief period of drying. The spores were surrounded by liquid containing ample

food and were kept in diffuse light, hence no haematochrome was formed although over a month had elapsed before germination took place. The proportion of successful germinations was very high, practically all the spores germinating within 48 hours after transference to the fresh solution.

(ii) Swarmers from a net obtained in culture of Miss Britten's Grahamstown material were put on agar, settled down and formed spores which developed well, many reaching a large size (56μ). After some weeks, some of the spores, now golden brown in colour, were removed from the agar, washed and transferred to 50 per cent culture solution. Germination followed and many proved to be zygospores, the contents dividing twice to form four zoospores (Fig. 2, e—h). In the larger spores however the first two divisions were followed by a third, resulting in eight zoospores of normal size (Fig. 2, j—l).

The germination of one of these larger spores was followed from an early stage. When first seen (3 p.m.) the spore was still almost round, with the wall just beginning to project at one point; the contents were already divided, the 8 zoospores clearly distinguishable (Fig. 2, j); by 3.27 p.m. the vesicle was developing and the contents gradually passing into it as it enlarged (Fig. 2, k). At 3.33 p.m. the vesicle, which had reached its full size, ruptured distally and the 8 zoospores (ranging from $10 \times 14\mu$ to $17 \times 21\mu$ in size) escaped one after the other and began to swim actively in the water. The zoospores had shown activity within the vesicle, but during the actual expulsion they were quiescent, movement being resumed after liberation (Fig. 2, l). Activity lasted less than half an hour—by 3.55 p.m. all save one had settled down and five minutes later that one too was quiescent. Polyhedra were soon formed, the processes thrown out in their formation being particularly well marked.

In this culture the spores were formed and matured on agar, reached a larger size than in Culture (i) and had developed a considerable amount of haematochrome and fatty reserve material. Both zygospores and azygospores were present but the former predominated.

(iii) Coenocytes from Groen Vlei were put on agar on 1st November; on the 4th, at 8 p.m., swarmers were being liberated in large numbers. Put on agar they developed rapidly and 10 days later some of the green hypospores were transferred to culture solution. The following day many polyhedra and zoospores were present and many more spores were still germinating. Here the majority were azygospores but many zygospores were also present. By the morning of the 17th many nets were already present, i.e. less than 13 days from the liberation of the swarmers. The germination of several azygospores was watched; the polarity of the zoospores was normal, the apex directed outwards, the reverse of Culture (i). In some cases the zoospore showed definite movement within the vesicle before rupture.

Here the parent nets were collected from the Flats, the swarmers were liberated and the spores developed on agar plates; the spores developed rapidly and were germinated while still comparatively small. The proportion of germinations was high and development extraordinarily rapid, the whole process from liberation of swarmers to formation of germ nets being completed in $12\frac{1}{2}$ days. This period was the shortest obtained in any of the experiments and probably represents the minimum time necessary to complete the process.

(iv) In June 1941 old nets were collected at De Klip and a few days later young and mature nets were brought in from Groen Vlei. In both cases swarmers were liberated freely, both in vlei water and in vlei water to which culture solution had been added. In some cases "clumping" was marked and conjugation took place freely; the planozygotes showed a marked tendency to collect on the side of the culture glasses away from the light. There was considerable variation in the size of the swarmers; in some cases a single coenocyte produced swarmers of very diverse sizes, others formed swarmers larger than the gametes originally described. These were not seen to conjugate, although in other cases swarmers of different sizes sometimes copulated. Many plate cultures were made with swarmers from both localities with varying results. In some the swarmers settled down quickly and in such cases the majority formed hypospores. In other cases many of the swarmers remained motile for a long time, sometimes even for two days; here there was usually a high mortality among the swarmers, many eventually disintegrating instead of rounding off to form spores. At the time the weather was cold and damp and development of the spores was slow; after two or three weeks however many of the spores were ready for germination and a number of germination experiments were carried out. The spores germinated readily even though quite small but in nearly every case,

although conjugation had been noticed among the swarmers, zygospores were either absent or very rare; in most cases no sign of division could be detected in any of the germinating spores. This suggests the possibility of a difference in the viability of zygotes and azygotes.

The variation in size of the swarmers was interesting, but it has not been possible to establish the existence of a constant difference in size either among the obvious gametes or between them and those swarmers which can form spores directly without undergoing conjugation. On the whole, the weight of evidence points to the conclusion that this species is isogamous and that swarmers may behave either as gametes or as accessory cells.

II. HYDRODICTYON RETICULATUM.

The discovery that the formation of hypnospores from swarmers without conjugation is of common occurrence in *Hydrodictyon patenaeforme* suggested the possibility of a similar phenomenon in *H. reticulatum*, particularly in view of observations recorded by Klebs (1896, p. 168). He states that among conjugating gametes he noted cases where, without conjugation, individuals settled down and formed minute rounded spores apparently identical with those formed by zygotes: he was unable to follow the fate of these spores as in his cultures they did not develop further.

Fortunately, material was to hand for experiments—that brought from Cambridge in 1936 was still flourishing, while Miss Britten's discovery of the species near Grahamstown provided a second strain for comparison; finally, material from California was also used. In the cultures all three strains were used at different times, treated similarly throughout and apart from minor variations in behaviour attributable to slight differences in individual cultures, proved to be identical both in structure and life history.

Previous accounts of the species differ markedly on certain points, notably: (1) The structure of the chloroplast, (2) details of the mechanism of net formation in asexual reproduction, and (3) the form of the germ net formed from the polyhedron. Hence in this study, in addition to the problem of the nature of the resting spore, special attention has been paid to these three subjects.

A. ASEQUAL REPRODUCTION

1. The Daughter Net.

(a) Size and Form.

The three strains have been cultured under varying conditions. The same culture media as were used for *H. patenaeforme* proved at least equally satisfactory for this species. The latter is indeed remarkably

tolerant, thriving under conditions which are unsuited to the two other species.

The form and size of the net are extraordinarily susceptible to external conditions. The size given in the majority of text-books as the maximum is far from representing the true facts: nets collected by Professor Oliver in Egypt were considerably over 30 cm. long although still quite young. In the present cultures good results have been obtained by isolating well formed young nets, newly liberated or a few days old, in large flat glass vessels, 3—4 in. deep by 12 in. in diameter, nearly filled with culture solution and covered with a glass plate. Thus the young net is isolated from a very early stage in a superabundance of nutritive solution and left undisturbed, preferably in a south window where the light is good, without direct sunlight. By this method perfect nets up to 30 in. or more in length have been obtained, although those grown in incinerated soil and tap water in glass biscuit barrels in Chelsea in 1936 (Pocock, p. 277) still hold the record for size. Of the two nets thus grown, the larger reached a length of 114 cm. by approximately 4—6 cm. in diameter, while the second was only slightly smaller.

It must be emphasized that, if large nets are desired, it is essential that the young net be isolated at once in a large volume of the nutrient solution and left undisturbed, since, once the net has reached a certain stage in development, the addition of even a small quantity of fresh liquid will act as a trigger mechanism precipitating daughter-net formation. Left undisturbed under such conditions, particularly in a shallow circular container, the net will develop evenly, spreading round the perimeter of the dish until it may reach a great size. Ultimately, however, all, or nearly all, of the constituent coenocytes may more or less simultaneously form daughter nets. In contrast, nets isolated in large beakers instead of such flat dishes develop less evenly, the part nearer the surface usually growing more rapidly; if, however, instead of the liquid nutrient a soil-and-water culture is used, this result may be reversed, the part of the net lying nearest the soil enlarging the more rapidly. In the latter type of culture, it is normal for some only of the coenocytes to form daughter nets, the rest either continuing growth unchanged or liberating swimmers.

The following examples will serve as illustrations: On the 25th November 1940 a single daughter net from the Heatherton Towers material was isolated when three days old and nearly 1 in. in length, washed and placed in a large glass dish of culture solution (Juller's "Volvox solution" with soil extract made from De Klip soil). On the 12th December the net was 28 in. long, one end slightly broader (3 in. diameter) than the other (2 in. diameter) and still showed no sign of daughter-net formation.

A little later a similar net 34 in. long was obtained and another of 30 in. still very young, only 1 in. wide, with coenocytes still small and immature.

(b) *Factors Influencing Development.*

External factors on which the rate of development directly depends are: (1) Food supply, (2) light, (3) aeration and (4) temperature.

Usually when a single well grown net is isolated in a large beaker of culture solution it soon produces innumerable daughter nets; if left undisturbed this results in a very crowded culture; the individual nets are small and show uneven development. As they are liberated they tend to float up to the surface and there form a dense mass, the upper part on the surface, the rest submerged. In many cases part of a net, often one end, touches the surface while the rest of it is below. The better aerated upper part of such a net develops more rapidly than lower down in the culture; if it is the end which is at the surface the result is a club-shaped structure, the head of the club often several times the diameter of the handle. If an intermediate part of the net is at the surface this enlarges similarly and the result is a population of nets variously distorted according to their position in the culture. Other nets, formed later than the majority, remain in the less crowded but also less well aerated water below the floating mass; these develop slowly, often hardly enlarging at all. If one of these unevenly developed nets is isolated while still young and transferred to a large flat dish of culture solution where all the parts are equally well aerated and well nourished, the whole net develops at a uniform rate and hence the aberrant form is retained. Thus nets of all shapes and sizes yet all of the same age may be obtained.

But if before the transference is made the nets have been left long in the original culture, the coenocytes though still small are mature and transference to fresh culture solution instead of stimulating growth in size, precipitates daughter-net formation despite the small size of the net and its constituent coenocytes. In such a case the daughter nets may vary enormously in size and in the number of coenocytes, since the number of zooids formed on division depends directly on the size of the dividing coenocyte.

Food supply being ample and aeration good, light can be shown to have a direct effect on the rate of growth. A few young nets, two or three days old, were taken from a vigorous single-net culture in which daughter nets had been formed, divided into two lots in which the nets were approximately equal in size and number and each lot put into a covered glass box of culture solution. One was left in a south window, where the light was good but exposure to direct sunlight avoided; the other was put on a shelf at the back of the room, much less well lighted. After

three days both sets were healthy and bright green in colour but the nets in the well-lighted window were nearly double the size of those in the poorer light. If, however, the experiment was long continued, the former began to show starvation symptoms, the colour became lighter and eventually changed from green to gold and the chloroplast showed extensive fenestration; those in the more diffuse light, though small, were still green and healthy. Addition of fresh culture solution restored the green colour and normal condition of the chloroplast within 48 hours, showing that the change in condition was due to the more rapid exhaustion of nutrient salts in the stronger light.

The rate of development is also directly affected by temperature; the cultures developed more rapidly in warm spring weather than in winter, though the optimum temperature seems to be fairly soon reached. Exact experiments on the effect of temperature were however not made.

(c) *Nets of Abnormal Form.*

In addition to the unevenness of growth due to differences in aeration, further abnormalities may arise in varying conditions. For instance, a high concentration of soil decoction, or a period of starvation followed by one of superabundance of nutrient, may in a young net result in uneven development of individual coenocytes; sometimes this seems to be in part at least due to changes in the wall at certain points so that when, on restoration of normal conditions growth is resumed, it takes place unevenly, resulting in beaded, hour-glass shaped, or otherwise unevenly shaped coenocytes. Once the coenocyte has assumed such an abnormal shape it retains it during its further development. Thus, since the daughter net takes the shape of its parent coenocyte, it follows that when net formation takes place in a culture of this kind, nets of all shapes, spherical, ellipsoidal, beaded, hour-glass or dumbbell shaped, may result. These, in turn, if cultured normally produce normal cylindrical coenocytes and eventually daughter nets of the usual cylindrical shape. The photographs reproduced in Pl. XVII, A, B, illustrate these points to some extent although the nets here shown were by no means extreme in their irregularity. Fig. A shows uneven coenocytes developed under crowded conditions followed by excess of nutrient solution resulting in well-formed cylindrical daughter nets (B) only very slightly irregular in shape. These figures should be compared with Pl. XII, where Figs. A and B show well formed cylindrical nets, while Figs. C and D show nets of various shapes from a crowded culture.

2. The Coenocyte.

(a) *Form of the Chloroplast.*

In a newly formed net, some 12 hours old, each coenocyte has a single

parietal band-shaped chloroplast, almost but usually not quite encircling the equatorial region of the fusiform cell, leaving the two ends colourless. In the chloroplast is embedded a single conspicuous pyrenoid (occasionally two are present), near which lies the nucleus (Fig. 5, a; Pl. XVIII, F). If conditions are favourable, development is rapid: the coenocyte elongates and soon becomes cylindrical, while the chloroplast increases in area soon completely encircling the cell and extending into the ends until it lines the whole wall. In form the chloroplast is a continuous sheet of green but even at a very early stage minute vacuoles appear in it, particularly towards the ends of the coenocyte where it is typically slightly thinner than elsewhere. These vacuoles or fenestrations may be seen under high magnification (normally examination with the immersion lens is necessary) as minute colourless spots in the apparently homogeneous green mantle covering the protoplast, sometimes scattered irregularly, sometimes ranged in short rows. If conditions are favourable and food supply ample these vacuoles remain small, but as development proceeds, if the supply of nutrient salts falls off or metabolism is otherwise hindered, they multiply in number, increase in size and coalesce so that the chloroplast becomes extensively fenestrated and ultimately may appear coarsely reticulate or even more extensively divided, particularly towards the ends of the coenocyte.

It must be emphasized, however, that such a "reticulate" chloroplast is most definitely a starvation phenomenon, whether resulting directly from actual deficiency of nutrient salts or from such other conditions as, for instance, too intensive insolation, as may upset the normal balance of metabolism. The *normal fully developed chloroplast is not reticulate* but even at an early stage it usually becomes minutely vacuolated or fenestrated (cf. Timberlake, 1901, p. 622). If nets showing the so-called "reticulate" type of chloroplast are removed from their unfavourable surroundings to fresh culture solution, the chloroplast may recover more or less completely. The photographs reproduced in Pl. XVI illustrate this very clearly. Nets in a crowded culture showed marked deterioration of the chloroplast which had become pale in colour and so attenuated in extent that in many places it was actually broken up into small distinct portions, appearing granulated (Figs. B and C). After five days in fresh culture solution the chloroplast was once more a continuous sheet but extensively fenestrated (Fig. D). Two days later recovery had continued so that in the greater part of the chloroplast the large fenestrations had filled up almost completely, lines of minute vacuoles marking their former position, except towards the ends of the coenocytes where large fenestrations still persisted (Fig. E). The two latter photographs are from nets placed with several others in fresh culture solution: the next one (Fig. F)

is from a net which was removed from the crowded culture at the same time, but was isolated from the others in a separate dish. Here, recovery had proceeded further; a few small vacuoles can be seen towards the ends of the coenocytes but elsewhere they have disappeared completely. Many small, newly formed pyrenoids are scattered among the larger old ones. In this case the first preparations for division were just beginning.

The crowded culture in question had been standing in diffuse light at the back of the room away from any window and the nets, though pale in colour, were still green. But often reticulation of the chloroplast is accompanied by a colour change to yellow, the chlorophyll being partly replaced by haematochrome. This may apparently be induced by two different causes: salt deficiency and exposure to too intense light. These two factors may work together or independently. If salt deficiency alone is in question, addition of fresh culture solution soon restores the green colour. Where the light factor is operative the matter is not so simple. Removal to a less strongly illuminated spot sometimes causes a reappearance of a green colour but must usually be supplemented by addition of nutrient solution. The intense insolation is probably, in part at least, an indirect cause, hastening katabolism and thus resulting in a premature exhaustion of the food supply.

If, as sometimes happens, such a coenocyte divides before the chloroplast has returned to its normal condition, the limits of the pigmented areas are the limits of the "pavement" and if a daughter net is being formed, the resultant net has large gaps in it. This fact is significant since it indicates that the chlorophyll is indeed diffused throughout the protoplast; the "reticulations" apply not merely to a chloroplast but to the protoplast itself.

The following experiment illustrates the effect of exposure to strong light. In late spring, two similar nets were isolated, one in each of two similar beakers containing equal amounts of culture solution; one was placed in the window in bright light, getting some morning sun, the other near the back of the room away from the window. At first both developed normally. Soon however a difference became apparent; while the latter continued to develop well, the colour being bright green, the coenocytes large and cylindrical, the former began to change colour, becoming pale golden, growth was checked and the chloroplast began to appear reticulate. Fresh culture solution was added and the beaker removed from the window. After two days the colour had already changed back to green and the chloroplasts had begun to recover. Similar results have repeatedly been obtained inadvertently.

(b) *Nuclei and Pyrenoids.*

In the young cell the position of the single nucleus is near the centre but to one side just within the chloroplast and adjacent to the pyrenoid

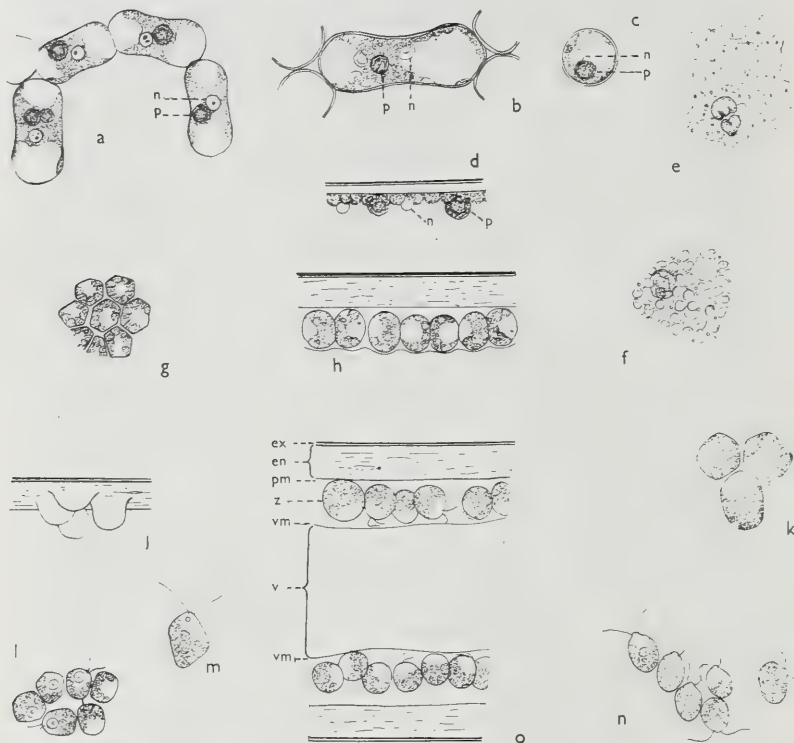


FIG. 5.—*H. reticulatum*. Structure of the coenocyte and daughter-net formation. a, cells from a young net, one nucleus (n), one pyrenoid (p), chloroplast girdle shaped; b, older cell becoming coenocytic, two nuclei, two pyrenoids, chloroplast extending into the ends of the coenocyte, already showing fenestrations; c, the same, end view, optical section; d, mature coenocyte, optical section showing two-layered wall, lined by the chloroplast, nuclei and pyrenoid projecting inwards; e, cleavage of protoplast beginning showing eroded pyrenoid, globules of food reserve; f, chloroplast broken up into granular plastids, nuclei showing between the granules, remains of one pyrenoid still present; g, cleavage complete, surface view of zooids (pavement stage); h, optical section, zooids formed, flagella not yet formed; i, peg-like ingrowths of wall; k, zooids beginning to separate, surface view; l, emission of flagella, some protoplasmic connection between cells still present; m, fully developed zooid; n, period of motility of the zooids; o, optical section of part of the coenocyte showing zooids in process of net formation, kept in position by the vacuolar membrane; ex, external layer of wall; en, inner, much swollen laminated wall; pm, outer protoplasmic membrane; z, zooids; vm, vacuolar membrane; v, central vacuole. d $\times 200$; a—c, k, m $\times 1000$; other figures $\times 750$.

(Fig. 5, a). As the cell enlarges, the nucleus divides and a second pyrenoid appears, formed *de novo* and not by division of the primary pyrenoid, and as growth proceeds pyrenoids and nuclei multiply apparently at first more or less at the same rate. Division of the nucleus may lag behind multiplication of the pyrenoids, for example, cells with two well-developed pyrenoids may still be uninucleate, but the reverse may also occur.

The nuclei and pyrenoids are not directly connected with one another, but usually a nucleus lies adjacent to each pyrenoid (Fig. 5, b, c).

The pyrenoid is large and completely enveloped in the chloroplast which consequently appears much thicker over the pyrenoids than elsewhere, projecting with the pyrenoid in towards the centre of the cell (Pl. XVI, A). As a consequence of this greater thickness of the chlorophyll-containing layer combined with the highly refractive substance of the pyrenoids, they usually appear as brighter green bodies in the chloroplast. It is only on examination with the immersion lens that an optical section of the pyrenoid can be obtained and the colourless central body be distinguished. This is surrounded by a broad border of deep bluish green, the whole outline being markedly angular. Very occasionally a vacuole in the chloroplast may occur above a pyrenoid, allowing the colourless centre to be seen. Such a case was observed in a young net in which the coenocytes had reached the two-pyrenoid stage; the net had been raised in somewhat crowded conditions and the chloroplasts, which did not yet line the entire wall, were granular and already showed occasional vacuoles (Fig. 5, b).

The nucleus is smaller than the pyrenoid and lies in the cytoplasm just inside the chloroplast, sometimes partly embedded in it but never surrounded by it as is the pyrenoid. In a fully normal coenocyte the density of the chlorophyll-bearing outer layer of the cytoplasm makes it very difficult to distinguish the nuclei; in less well nourished coenocytes, where the chloroplast is extensively fenestrated, many nuclei can be seen through the small vacuoles in the chloroplast. There is a nucleolus (cf. Proskauer, p. 403) similar to but less conspicuous than that characteristic of the Volvocaceae. The relation of pyrenoids and nuclei to one another and to the chloroplast is best seen in optical section of the coenocyte, though even there the nuclei, lying within and against the chloroplast, are only distinguishable with difficulty, whereas the pyrenoids in their mantle of green, projecting well into the cell, show up clearly.

3. Changes Preceding Division.

(a) *Methods of Investigation.*

It was found that both daughter-net formation and liberation of swarmers took place readily and, so far as could be judged, with complete

normality, on agar, and as individual coenocytes on agar could easily be kept under observation. plate cultures have been used extensively in following the changes in the protoplast which culminate in the formation of motile cells, always confirmed by comparison with material in liquid cultures. Here, as in *H. patenaeforme*, the best results were obtained by using 0.75 per cent agar made up in the normal culture solution. Single coenocytes or small fragments of nets were placed on the agar in a drop of culture solution and examined from time to time. The water immersion lens was used direct or a cover slip lowered over the coenocyte under observation and the oil-immersion lens used. Lower powers of magnification are insufficient for detailed observation of the changes in the protoplast. Treated thus, the selected coenocyte can be watched continuously without injury throughout the process of division, the cover slip being raised and removed without displacing the coenocyte. With liquid cultures coenocytes were mounted on slides for observation. The use of agar as a medium has the added advantage that under the slight pressure of the cover-slip, the coenocyte sinks into the agar in a slight depression.

(b) *Changes in the Protoplast.*

The first obvious sign of approaching division is an intensification of the colour of the coenocyte as the chloroplast becomes darker and congested in appearance, while the outer membrane of the wall, which in the vegetative state is closely adpressed to the protoplast, is now separated from it by a clear zone, particularly obvious towards the ends (Pl. XVIII, A); this is caused by the swelling of the inner layers of the wall. Under high magnification it can be seen that changes are already taking place in the protoplast: minute vacuoles have become abundant but the parts between them instead of appearing a clear homogeneous green have become

PLATE XVI. *Hydrodictyon reticulatum*. Cambridge culture.

The Protoplast.

- A. Young well-developed coenocyte, optical section, showing peripheral pigmented protoplast with pyrenoids projecting inwards(1009). $\times 800$.
- B. Part of starved net from crowded culture; pigmented region pale in colour, broken up into granules with many gaps. Note irregularity in union between coenocytes(1054). $\times 140$.
- C. Centre of B enlarged to show unusual union, attenuated granular chloroplast and large non-pigmented areas(1055). $\times 375$.
- D.—F. Nets from same starved culture transferred to fresh culture solution; D, and E, several nets in same culture vessel. F, Single net isolated in large quantity of culture medium:
- D. After three days, "granules" have coalesced but chloroplast still extensively vacuolated(1056). $\times 375$.
- E. Ends of three cells from same culture two days later; chloroplast now continuous, finely fenestrated except near end walls where it is still vacuolated(1060). $\times 510$.
- F. Cells from isolated net after three days. Chloroplast continuous with fine fenestrations right to end in upper coenocyte, with small vacuoles in lower. (1057). $\times 375$.

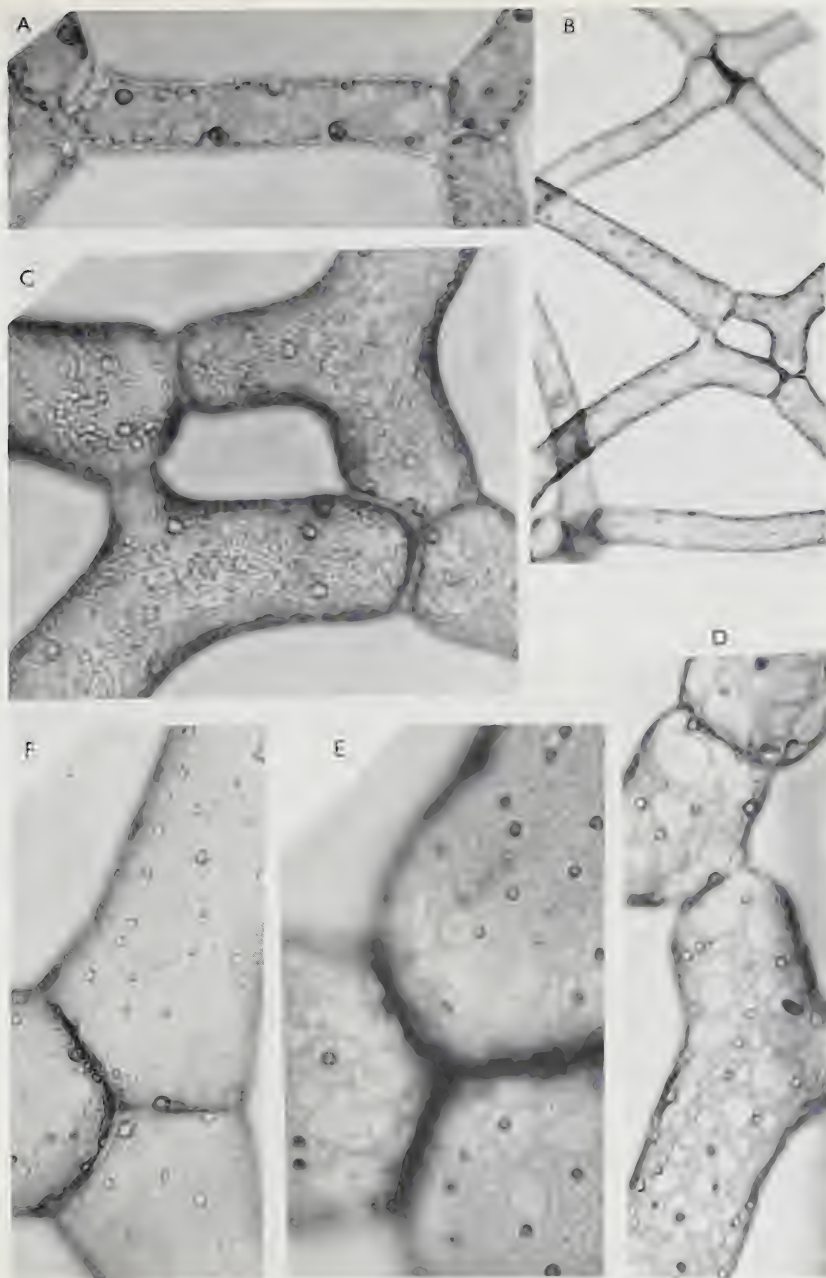


PLATE XVI *Hydrodictyon reticulatum*.

granular. Treatment with iodine shows that the granules are the beginnings of starch accumulations which stain very faintly blue. If the chloroplast is much vacuolated these areas are small and each may contain only one such starch centre; if however the continuous parts are extensive (as they would be in well nourished nets) each may show two, three or more granules. The intensification of the green colour, which is so sure a sign of approaching division, is caused by this granular structure. The stroma starch is not in definite grains; the appearance is rather that of a weak colloidal solution of starch, beginning at definite centres and growing progressively stronger as development proceeds.

The small vacuoles or perforations in the chloroplast multiply and tend to coalesce, forming a series of roughly parallel curves running this way and that and as in *H. patenaeforme*, giving the chloroplast at this stage a highly characteristic appearance like the whorls of a finger print (Pl. XVII, C), already noticed by Artari (1891, Fig. 1). Later, the vacuoles merge into one another forming continuous lines which divide the chloroplast into irregularly lobed parts (Fig. 5, e). In and between these parts there are sometimes small globules of volutin showing Brownian movement but these are not always present. Meanwhile the pyrenoids have begun to lose their clear outline (Pl. XVII, D), get smaller and break up rather as though they were becoming corroded from the outside until they finally disappear entirely. Division of the chloroplast has continued until it is completely broken up into small, closely packed, rounded or ellipsoidal bodies, each containing a central accumulation of starch (Fig. 5, e, f). By now the disintegration of the pyrenoids is usually complete and their starch content, very high before these changes began, has been evenly distributed throughout the chloroplast, in other words "pyrenoid starch" has disappeared and "stroma starch" has replaced it. The number of granules and globules of various reserve materials, some appearing hyaline and others blue-green by refraction, varies considerably according to external conditions.

The changes in the chloroplast have been accompanied by nuclear activity. Almost before division of the chloroplast has begun, nuclear division has speeded up and the resultant nuclei, now far more numerous than the pyrenoids, are distributed evenly throughout the protoplast and when chloroplast division is complete, the coenocyte presents a very characteristic appearance similar to that already described in *H. patenaeforme*, the whole protoplast finely granular with here and there a vestigial pyrenoid which has not yet wholly disappeared (Pl. XVII, D). Here, too, by varying the focus with the immersion lens, it can be seen that throughout the protoplast are evenly spaced colourless centres, each containing

a single nucleus, completely surrounded by a mantle of rounded granular plastids (Pl. XVII, E).

Cytoplasmic cleavage begins, at first dividing the protoplast into large irregular blocks, then into progressively smaller portions until the whole protoplast is divided into small prisms of equal size, polygonal in cross section: although the majority of these are hexagonal in section, some may be pentagonal, while other forms may also occur. In general, the better nourished and developed the dividing coenocyte, the more regular is the division (Pl. XVII, F; Pl. XVIII, B). During the process of division there is a slight thickening of the protoplast so that the prisms are longer ($7-9\mu$) than the thickness of the vegetative protoplast. This stage, on completion of cleavage, is the well known "pavement" stage of Pringsheim and a surface view at a focus slightly below the actual surface of the protoplast shows it divided up into approximately equal polygonal areas, from $6-9\mu$ in diameter. Some of the progressive changes which culminate in cleavage have already been described and figured by Klebs (1891, p. 795, Taf. IX, Figs. 12, 13) and Oltmanns (Ed. II. V. 1, p. 279, Figs. 187, 1-4).

Meanwhile changes in the wall have continued; even at low magnifications it now appears conspicuously thicker, the thin outer membrane separated from the protoplast by a colourless hyaline zone formed by gelatinization of the inner layers of the wall; apparently enzymes secreted by the protoplast during preparation for division act on the inner layers, the outer membrane remaining unaltered.

In old coenocytes peg-like thickenings of the wall projecting inwards and depressing the protoplast are of common occurrence (Fig. 5, j; Pl. XVIII, B). They are laminated, highly refractive and often complex in form; in surface view they appear as clear patches in the chloroplast. Such "pegs" remain unaltered, the substance of the inner zone of the wall swelling up round them. Similar peg-like ingrowths of the wall were described by Iyengar (1925, p. 135) in *H. indicum* but are comparatively seldom seen in *H. patenaeforme* (but cf. Fig. 4, e, f).

The sequence of changes described above is the same for all dividing coenocytes, whether they are going to form daughter nets or liberate

PLATE XVII. *Hydrodictyon reticulatum*, Cambridge culture.

Daughter-net formation.

- A. Part of nets from old crowded culture showing irregular coenocytes(1006). $\times 37$.
- B. Net formation in same culture after transference to fresh culture solution (1073). $\times 57$.
- C. Preparation for division, "whorls" (on agar)(1058). $\times 375$.
- D. Chloroplast broken up into granules, pyrenoids eroded(1008). $\times 800$.
- E. Pyrenoids gone, granular chloroplasts arranged round evenly distributed nuclei, ready for cleavage(1059). $\times 375$.
- F. Cleavage nearly complete, "pavement" stage(1062). $\times 375$.

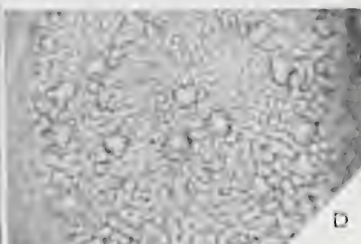
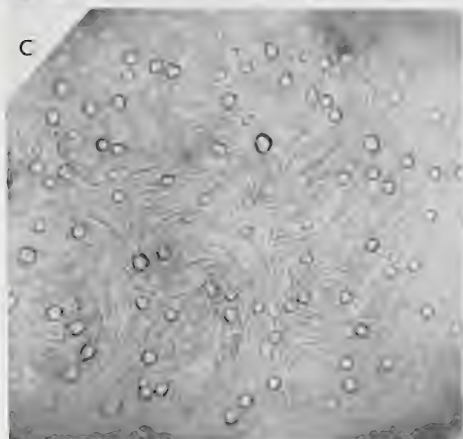
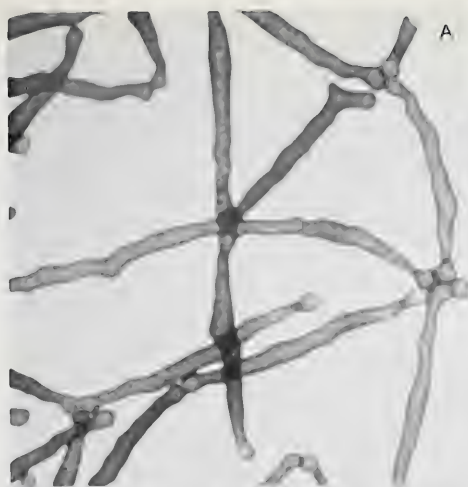


PLATE XVII. *Hydrodictyon reticulatum*.

gametes; the only difference is that, in the latter case, nuclear division and subsequent cleavage of the protoplast go one step further so that the resultant parts are smaller. Further, on the whole, there is no material difference in the behaviour of this species and that of *H. patenaeforme*; there are slight differences, very difficult to define and more of degree than of procedure; for example, there appears to be some difference in texture, that of the latter species being rather coarser than in *H. reticulatum*.

(c) *Time of Further Development.*

The pavement stage having been reached, differentiation of the motile cells may follow immediately or the coenocyte may remain quiescent for some time. The preliminary changes, too, may take place rapidly, being completed in a few hours or they may take some days. Various factors seem operative, some internal, e.g. the condition of the net, and this does not mean merely its actual age though that is undoubtedly important, others external. As a general rule, the preparatory stages are completed during the late afternoon or evening while the formation of the zooids or gametes culminating in their activity most often takes place in the early hours of daylight (7—9 or 10 a.m.), although this is not invariably the case.

Coenocytes in the pavement stage have been watched through the night; in every case no net formation or liberation of gametes took place until the following morning between 8 and 9 a.m. In other cases, coenocytes on agar remained in the pavement stage for several days. Probably the time of net formation depends on several external factors,* of which light seems to be the most important. Keeping the mature coenocytes in the dark may delay net formation for a few hours. For example, agar plates with coenocytes in the pavement stage put in a dark cupboard overnight and kept there until 11 a.m., started net formation on being brought into the light. On the other hand, if left in the dark, it was found that although net formation was delayed for some hours it eventually took place quite normally even in the absence of light.

Since exposure to direct sunlight for a few minutes sometimes served to induce movement, it is possible that in nature net formation normally occurs early in the morning, soon after sunrise.

4. Formation of the Daughter Net.

(i) *The Zooids.*

(a) *Differentiation.* If development in a net-forming coenocyte is closely watched, the first sign of approaching movement is a change in

* Cf. observations of Jost (1930, p. 60-61).

the appearance of the polygonal areas which begin to lose their angular shape while the colourless central zone changes its position, coming to lie at one side of the polygon (Fig. 5, g). This is the first part to become rounded and will form the colourless apex of the zooid, and since it usually lies sideways as the zooid is differentiated, it follows that the polar axis of the latter lies more or less parallel to the wall of the coenocyte or inclined at a slight angle to it. The zooids are now mature and ready to separate (Fig. 5, g—k; Pl. XVIII, C), but since in a healthy well-nourished coenocyte they form a closely packed layer lining the whole wall of the coenocyte, ends included, space must be made before movement can begin.

(b) *Separation.* The necessary space is obtained by a simultaneous swelling of wall and protoplast accompanied by a slight shrinkage of the central vacuole while the shape of the individual zooids also alters slightly as emission of the flagella begins.

Careful measurements at the two ends and the centre of net-forming coenocytes taken at intervals, starting with the pavement stage, show that as movement begins there is an increase in diameter and also in the length of the coenocyte, the gelatinization of the inner layers of the wall causing the outer membrane to stretch. At the same time there is also a slight increase in the external dimensions of the protoplast as movement begins. At first this increase is just sufficient to allow for the rounding off and the first vibratory movement of the zooids, but as movement strengthens and the separation of the zooids continues, so also does the increase in diameter of the whole protoplast.

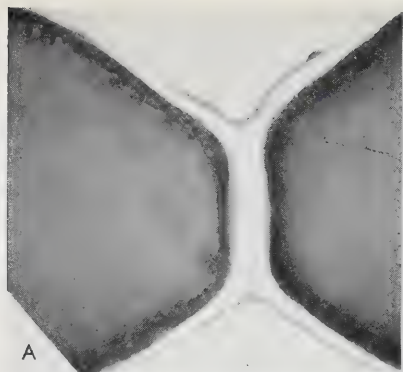
The zooids now form a dense green layer confined between two very delicate membranes, both cytoplasmic in origin. The one on the outside, the protoplasmic membrane, abuts on the gelatinizing inner wall layers, the other, on the inside, is the membrane surrounding the central vacuole of the coenocyte. Treatment with methylene blue shows up both mem-

PLATE XVIII. *Hydrodictyon reticulatum*. Cambridge culture.

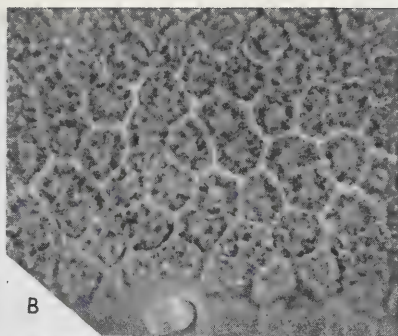
Daughter-net formation—*continued*.

- A. Ends of two adjacent coenocytes; on the left, pyrenoids showing, on the right, "pavement" stage. Thickened wall at junction clear(1063). $\times 375$.
- B. Pavement stage, cleavage nearly complete, same cell as Plate XVII, F, a little later in development. Large "peg" on wall at bottom(1064). $\times 800$.
- C.—E. Net formation, living, unstained, on agar. C. Zooids differentiated(1024).
- D. Movement about to commence, zooids beginning to separate(1025).
- E. Movement just ended, zooids arranged in net formation, attachment of cells very light, easily broken. Chloroplasts still many, granular(1026).
- F. Coenocytes from day-old net, chloroplasts coalesced to form a continuous girdle-shaped band(1028).
- G. Slightly older net, chloroplast beginning to extend into ends of coenocytes (1029).

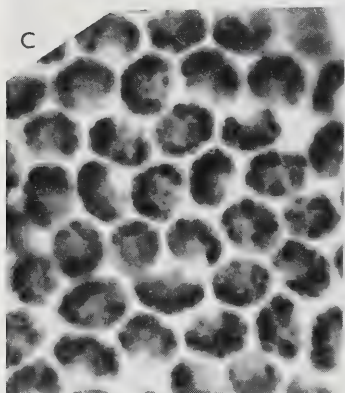
C.—G. $\times 1100$.



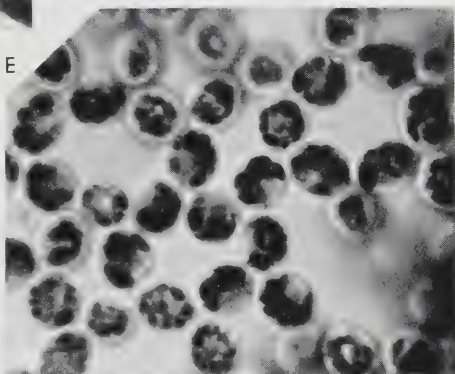
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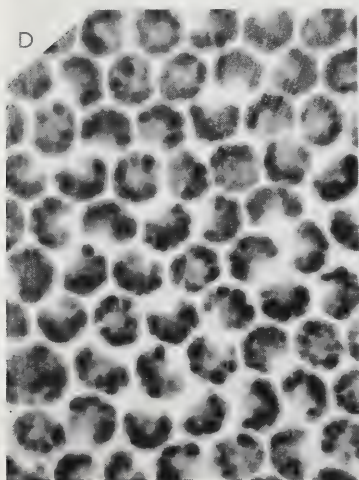
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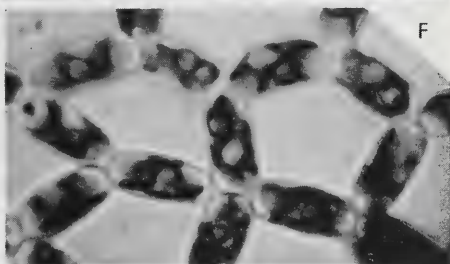
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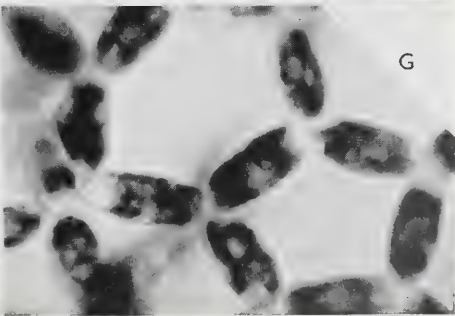
E



D



F



G

PLATE XVIII. *Hydrodictyon reticulatum*.

branes. At first only the wall is stained, the surface membrane deep purple, the mucilaginous inner layers light purple, but as the stain penetrates further, both cytoplasmic membranes also stain purple, the outer darker than the inner wall layers, the vacuolar membrane a more delicate but quite distinct shade. The persistence of the vacuolar membrane throughout the actual process of the formation of the net is a most important factor in determining its form (Fig. 5, o).

MEASUREMENTS OF A NET-FORMING COENOCYTE.

(a) At 10 a.m. at commencement of movement. (b) At 11 a.m. on completion of net formation, taken at corresponding points near the two ends (1 and 3) and in the centre (2).

	Outside diameter.			Diameter of protoplast.			Thickness of wall.		
	(1)	(2)	(3)	(1)	(2)	(3)	(2)	(2)	(3)
(a)	147 μ	97 μ	119 μ	139 μ	89 μ	106 μ	4 μ	4 μ	6.5 μ
(b)	163 μ	111 μ	142 μ	150 μ	93 μ	124 μ	6.5 μ	9 μ	9 μ

(c) *Form of the Zooid.* As movement began, careful examination was made with the immersion lens of both living and recently fixed material. For the latter, coenocytes in which movement was strong were mounted on slides and killed with iodine in potassium iodide to reveal details of the structure and behaviour of the zooids and their relation to one another which might not be discernible in the living state.

Each zooid has a clear apex from which arise two delicate flagella, less than body length even at their fullest development. The nucleus, lies below the apex somewhat to one side and one contractile vacuole could often be distinguished near the point of insertion of the flagella (Fig. 5, m); here again careful search failed to establish the presence of more than one vacuole save in very rare cases, but it is probable that there are actually two lying one behind the other and pulsating alternately. Several rather large discoid chloroplasts line the posterior two-thirds of the cell and among them are usually one or more slightly smaller highly refractive granules or globules. There is no eyespot.

In a normal healthy coenocyte the zooids show marked uniformity in size (5.5 x 7 μ to 7 x 9 μ), but cases were occasionally observed where the size varied considerably, a few of the zooids being very much larger than the majority (Fig. 5, k, o). This probably results from the omission of the final nuclear division, or in the case of some monstrous zooids of several divisions, in the part concerned and is caused by abnormal conditions leading to irregularity in development.

The flagella could sometimes be seen quite easily in living zooids but were much more clearly distinguished after treatment with iodine.

Although rather short, as might be expected from their very limited time of activity, they are less fine in proportion to the size of the zooids than are those of the zoospore produced by germinating hypnospores (Fig. 5, 1—o).

(ii) *Net Formation.*

(a) *Movement of Zooids; Persistence of Vacuolar Membrane.* Movement is initiated by a spasmodic quivering here and there among the developing zooids. This soon becomes general; as they free themselves the zooids jostle one another and as the space confining them is gradually increased they begin to move about relative to one another. At first neighbouring zooids may still be in protoplasmic connection: here and there delicate protoplasmic strands linking them may sometimes be seen (Fig. 5, 1), but as movement grows stronger these disappear entirely and the zooids move about comparatively freely (Fig. 5, n. o), pushing against one another, turning on their axes and even occasionally changing place relative to one another, but always maintaining their parietal position in the coenocyte. Movement remains restricted, particularly in the direction perpendicular to the surface of the coenocyte: it is as though the zooids were moving over the surface of a cylinder within the wall of the coenocyte and of smaller diameter than the latter and this is, in fact, the case, since the vacuolar membrane persists preventing movement inwards away from the wall. In the vegetative state the space between the vacuolar membrane and the wall is completely filled by the protoplast; as the zooids are differentiated within this space it enlarges slightly mainly owing to the greater enlargement of the wall of the coenocyte, while it is probable that a slight contraction of the central vacuole during net formation also contributes to the increase in space between the two cytoplasmic membranes (Fig. 5, o).

So delicate is the vacuolar membrane, however, that it is often impossible to distinguish it through the dense outer layer of green zooids: treatment with iodine stains it brown and makes it more distinct, and by careful focusing with the immersion lens it is sometimes possible to see it here and there through gaps between the zooids, even in normal coenocytes with their typically dense contents. In nets which have reached a fair size and then undergone a period of partial starvation, the protoplasmic contents may be interrupted by more or less extensive vacuoles: consequently, when net formation supervenes the number of zooids is much smaller in proportion to the size of the coenocyte than is usually the case. In such coenocytes gaps, corresponding to the vacuoles, form in the layer of zooids and consequently in the daughter net, and as a result the vacuolar membrane may show clearly through the gaps.

Further, in such conditions the vacuolar membrane is sometimes thicker than usual. One culture for instance showed this well, particularly as expansion of the outer wall had been greater than usual during the early stages of net formation; in some coenocytes the central vacuole instead of being continuous had evidently become divided into distinct parts. Consequently, when net formation took place in many coenocytes the resultant net was incomplete and broken up into separate sections which remained adhering to the appropriate parts of the vacuolar membrane. Thus in one case the central vacuole had been divided into two so that the membrane formed two distinct sacs; when net formation took place two separate daughter nets were formed within the one coenocyte. Curious developments may result from such abnormalities in the form of the vacuolar membrane; constrictions are not uncommon, sometimes complete, at others only partial; in the latter case instead of distinct nets, a single net made up of a series of fusiform parts connected with one another by narrow necks may result. Nets formed in this way have the same appearance as those formed from beaded coenocytes referred to above although they have arisen in a different manner. Here they are contained in a normally cylindrical wall, whereas when they are formed in a beaded coenocyte, the wall itself is also beaded.

(b) *Reunion of the Zooids.* In addition to the restriction of free movement imposed on the zooids by the persistence of the vacuolar membrane, there appears to be some form of mutual attraction among them so that although they are in one sense separating during their period of motility, they yet maintain close association with their immediate neighbours, even though actual protoplasmic connection no longer exists. As movement strengthens the mutual jostling brings about a rearrangement, resulting in the formation of larger spaces in the mass of zooids made possible by the continued expansion of the wall. This in turn is undoubtedly controlled by the activity of the zooids themselves; it seems beyond question that the changes in the wall are brought about by the action of enzymes secreted by the zooids in their state of concentrated activity. If in unfavourable conditions this secretion fails and consequently the progressive expansion of the wall does not take place properly, the zooids are unable to separate sufficiently and all kinds of abnormal fusions result, such as are described by Jost (Fig. 15, etc.) and other workers.

The zooids, angular and approximately isodiametric to begin with (Pl. XVIII, C), become more or less rounded but show constant slight metabolic changes in shape during the process of net formation. The flagella though short are active, with the large contractile vacuole con-

spicuous near their insertion and to one side of the clear, broadly rounded apex (Fig. 5. m), the distinct chloroplasts still clearly defined in the posterior part (Pl. XVIII, D).

The zooids twist and turn, jostling one another in a curious fussy way. first one side, then another flattening as it comes in contact with another zooid; occasionally a zooid will suddenly go for a little excursion among its fellows, then return to its former position, or it may turn right round, at the same time twisting on its axis, so that it is abundantly clear that at this stage no protoplasmic connection exists between the zooids. Eventually the zooids resume the "settling in" motion, and grouping, most often in threes, becomes marked. Finally, movement slows and finally ceases, the sides in contact flatten, the part between them becoming angular and lengthening so that the two sides meet at an angle. This pushing out of the protoplast into the angle between the zooids is very pretty to watch; the whole process of "setting to partners" has a curiously purposeful appearance and is soon completed. Throughout, the apex is kept clear of contact and as the final arranging takes place the flagella are very active and the zooid pivots back and forth on the axis between the two points of contact—now slightly longer than the transversely placed polar axis—the pivoting serving to push the angular projecting sides well into place; in short, the zooid backs into place in two opposite directions at once. The photographs of successive stages in net formation reproduced in Pl. XVII, Figs. B—E, help to elucidate this description of a very delicate and beautiful phenomenon.

At this stage protoplasmic union is usually finally effected; sometimes it is possible to see delicate hyaline strands on each side of the angular projection near the centre of the contact-surface uniting the two protoplasts. Such strands are comparable with those put out by the zoospore as it changes into a polyhedron, but the zooids are so close together when they unite that it is seldom possible to see them. It is only in abnormal cases where the zooids have separated slightly after making contact, or have made contact when further apart than usual, that they become visible. In germ-net formation where the number of zooids is so much smaller they are more easily observed than in daughter-net formation. Since each zooid usually unites with two others at two opposite poles, there would appear to be a tendency for these processes to be produced in pairs at each side of the zooid. It is difficult to determine whether such connecting strands actually bring about union or whether they arise in response to a contact stimulus between two zooids after they have come into apposition. Some support is given to the first alternative by the formation of "horns" on zooids which have made contact with only one fellow zooid, since it would seem that such horns result from un-

satisfied contact processes. They are rare in normal daughter nets since when well formed there are in such nets no loose ends left, but are of common occurrence in germ nets, particularly in the edge cells of flat nets (Pl. XV, E; cf. Pocock, p. 270, etc.). Sometimes however union appears to be effected over the whole surface of the areas originally in contact.

Once contact has been established between the zooids, union becomes close; movement ceases and the flagella are withdrawn. The contractile vacuoles may persist a little longer, then they too disappear. In this condition (Pl. XVIII, E) the net is a very delicate entity and is easily made to disintegrate—slight pressure on the cover slip may destroy it as a net, causing the constituent cells to separate. If this happens, they cannot reunite, but if not injured may develop separately into coenocytes.

(c) *Changes in Zooids following Union.* Once union has been effected, the zooids round off contracting slightly as they do so, and the spaces between them increase in size, forming the meshes of the net (Pl. XVIII, E). Normally wall formation begins immediately net formation has been completed and is accompanied by changes within the protoplast as well as in the shape of the cell. Initially rounded, the cell soon begins to elongate, becoming first barrel-shaped or fusiform and eventually cylindrical. Within the protoplast the changes follow the normal sequence found within the genus wherever a motile cell comes to rest and enters a period of vegetative growth, that is, first the separate chloroplasts, still distinguishable in the cells of the newly formed net, reunite to form a single parietal chloroplast, and from the position of the colourless apex of the zooid in relation to the uniting "poles" (actually at opposite ends of an equatorial diameter of the zooid, not the true poles) it is obvious that this chloroplast must be more or less median in the cell and not at first completely encircling it.

As the chloroplasts coalesce, a pyrenoid is reconstituted in each cell typically at first one per cell, occasionally two (Fig. 5, a; Pl. XVIII, F). Elongation of the cell along what was the equatorial diameter of the zooid, initiated at the time of union, continues, the ends becoming more and more drawn out until there is a colourless zone on each side of the chloroplast as the cell becomes cylindrical (Pl. XVIII, E—G); the chloroplast continues to increase in extent until it lines the whole wall, the ends of the coenocyte at first excepted.

(d) *Rupture of the Coenocyte Wall and Accompanying Changes.* Returning to the parent net:—while the daughter net has been forming, the changes in the wall of the parent coenocyte have continued. It normally

remains intact until after net formation has been completed. Once this is achieved, however, gelatinization of the inner layers proceeds apace, the limit of tensile strength of the now much attenuated surface membrane is reached and as the daughter net elongates the wall ruptures, the recoil often throwing it into folds so that it wrinkles like the fingers of a glove. Most often rupture is near the centre of the coenocyte (Pl. XII, A, two central nets), but it may take place near one (left hand net in the same figure) or both ends of the coenocyte, or irregularly. In the last case it usually disintegrates at once: more usually its remains persist for some time while even after it has disappeared or the net slipped out of it, the inner layers of the wall, becoming progressively more dilutely mucilaginous, may form a protective zone round the young net (Pl. XII, B—D) until finally the last traces disappear. The mature net is not noticeably mucilaginous.

(e) *Time taken in Daughter-net Formation.* As in the case of the preliminary changes, so in actual net formation, the rate of development varies considerably and is largely dependent on external conditions. The whole process may be completed within a few hours or may be delayed considerably.

In one experiment a number of coenocytes, either already in the pavement stage or approaching it, were isolated, some in culture solution the rest in distilled water, at 10 p.m. (3rd November 1939). By 9 a.m. the following morning not only had all the coenocytes in both cultures formed daughter nets, but the constituent cells of the nets already had band-shaped chloroplasts and well-formed pyrenoids. Here, separation of the zooids, movement, reunion and reorganization of the protoplasts, including formation of pyrenoids, had all been completed in less than 12 hours. The time taken in the actual formation of the net was of course very much less.

5. Abnormalities in Net Formation.

(a) *Rupture of the Vacuolar Membrane.*

Cases have been observed in which the vacuolar membrane had been ruptured before the daughter net was fully formed. The result was chaos; the zooids, no longer kept in place by any inner control, crowded inwards and junctions were effected in haphazard fashion, often several together, as many as five to seven (Pl. XVII, A), while many united by one end only, failing to make contact at the opposite end.

Probably many of the abnormal nets met with in old or crowded cultures are the direct result of injury to or deterioration of the vacuolar membrane.

(b) Part Only of the Protoplast Used.

Occasionally cases were encountered where only a part of the protoplast was used in the formation of the net. These cases are usually either pathological or traumatic, the result of interference (as, for example, by undue pressure of the cover slip) at the critical period of net formation, that is, either during the period of motility of the zooids or immediately after union has been effected and before the cells have become firmly attached to one another. Nevertheless, such cases are of interest in their implication, since they may serve to illustrate the plasticity of the motile bodies.

Instances of pathological interference were occasionally obtained where coenocytes had been kept long on agar and the culture had been badly contaminated. Coenocytes attacked by fungus were common in such cultures; usually the whole protoplast was affected and soon degenerated, but in a few cases one end only was attacked while the rest of the protoplast remained unaffected, continued to develop and even formed a daughter net in the usual way.

Traumatic interference gave a different picture, since here the parts of the protoplast were all completely healthy, but normal development was interfered with by some external agency. In the experiments concerned this was either inadvertent pressure on the cover slip or rough handling with needles, the result in both cases being the rupture of the wall of the parent coenocyte. The degree of interference depends to some extent on the stage in net formation already reached. In one case, most of the zooids had completed net formation, probably they had already united before the accident happened, but some near the torn end had failed to unite and these escaped into the liquid outside the parent. Some of them soon degenerated but others continued to develop, some singly or united together in small groups, and formed coenocytes of normal structure though aberrant shape, while a few rounded off and formed resting spores which proceeded to develop on the agar as normal hypnosporos.

If pressure is applied to a very young net in which the zooids have only just come to rest and have therefore not yet secreted walls, it is not difficult to induce separation of the cells. In this case, too, the separated cells may degenerate or may continue to develop independently, usually directly into coenocytes more or less rounded in form, each of which may eventually form a daughter net, or sometimes into hypnosporos. The value of agar-plate culture is very apparent in all such cases as the separated cells can be kept under observation and their development watched. In this species such individual coenocytes almost invariably ultimately produced daughter nets.

B. SEXUAL REPRODUCTION.

1. The Gamete.

(a) Formation and Behaviour.

As has already been stated, the early stages in gamete formation are identical with those preceding the formation of daughter nets, but nuclear division and consequently protoplasmic cleavage proceed a step further: in the pavement stage therefore the polygonal areas are smaller and the number of gametes proportionately greater. Maturity of the coenocyte is again heralded by changes in the wall accompanying the formation of the tiny swimmers; again the changes consist of the thickening of the inner layers, most marked towards the ends of the coenocyte, causing stretching of the surface membrane with increase in the size of the coenocyte. Here, too, there is an appreciable though slight increase in the size of the protoplast. From now on, however, differences in development begin to appear. Apparently there are two somewhat different ways in which further development may proceed:—

(i) The diameter of the coenocyte ceases to increase while that of the protoplast begins to decrease. As in the case of net formation this was demonstrated by actual measurements of gamete-forming coenocytes at intervals in the hour preceding liberation of the gametes. The contraction of the protoplast marks its break-up into swimmers which round off, separate from one another as they do so and become very active, and appears to be due to a contraction of the vacuolar membrane as the swimmers are differentiated. This widens the space between the mass of green swimmers and the wall, giving the appearance of continued swelling. This is probably purely mechanical: as the internal pressure is relaxed the inner layers of the wall, already gelatinized, bulge inwards towards the contracted mass of swimmers.

MEASUREMENT OF A GAMETE-FORMING COENOCYTE.

(a) At noon. (b) At 12.20 p.m. (c) At 12.50 p.m., when movement was beginning, while at 12.55 p.m. the gametes began to escape. In each case 3 measurements were taken: 1 and 3 near the ends, 2 near the centre of the coenocyte.

	<i>Diameter of coenocyte</i>			<i>Diameter of protoplast</i>			<i>Thickness of wall</i>		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
(a)	177 μ	124 μ	164 μ	164 μ	111 μ	148 μ	6.5 μ	6.5 μ	8 μ
(b)	190 μ	129 μ	173 μ	177 μ	115 μ	154 μ	6.5 μ	7.0 μ	9.5 μ
(c)	208 μ	129 μ	177 μ	164 μ	75 μ	142 μ	22.0 μ	27.0 μ	17.0 μ

(ii) In the second case, instead of the vacuolar membrane collapsing it persists and may even increase slightly in size, carrying the swarmers outwards on its surface. When this happens the wall continues to expand and the outer membrane ultimately ruptures in several places; as the expansion may be unevenly distributed in such cases the coenocyte may be thrown into a series of bulges. This condition is ideal for studying the vacuolar membrane and the structure and movement of the swarmers. The expansion of the membrane separates the swarmers slightly; they are fully formed but though motile have not yet reached the peak of their activity. Examination with the immersion lens shows the gametes moving over the surface of the central vacuole, or rather, its membrane, with a curious creeping motion, partly flagellary, partly metabolic. As the gametes move about, patches of the vacuolar membrane show clearly between them even though unstained. Delicate markings, the imprints of pyrenoids and the inner surface of the protoplast, show here and there. In the swarmers themselves it is possible to study details of structure more easily than later when they have reached the height of their activity; in particular, the contractile vacuoles show clearly. Here at last two could often be distinguished; as one approached anastole, the second was usually just beginning to appear, thus confirming what was previously mostly surmise, that there are in fact two contractile vacuoles, certainly in the gametes and probably in all types of swarmers. The action of the flagella too could be studied more easily (Fig. 6, a, p).

(b) *Liberation.*

In either case, movement of the swarmers soon grows stronger and eventually the wall of the coenocyte is ruptured, sometimes near the centre, more frequently near one end, usually where the thicker terminal zone of the wall ends. Escape may be directly into the water or a delicate membrane, probably formed by part of the protoplasmic membrane enclosed in mucilaginous material from the inner layers of the wall, protrudes, forming a vesicle into which the swarmers pour, the vesicle enlarging progressively, the outer mucilaginous envelope becoming more and more attenuated until finally the increasing pressure of the escaping gametes ruptures the membrane and they swarm out into the water.

The latter method of escape was most beautifully seen in the summer of 1938 in a rather old culture of the Cambridge material. The culture was a crowded one, in a large oblong glass trough; looking down into it one morning peculiar light coloured filmy excrescences, more or less bulbous in shape, were noticed on a large number of the coenocytes, in all cases near the centre of the coenocyte concerned. On examination these were found to be masses of swarmers held together in a mucilaginous

film and constantly increased by an unending stream of swimmers issuing from the coenocyte, until finally the membrane ruptured, the mass broke asunder and the swimmers streamed out into the water. Myriads of swimmers were liberated into the water of the trough but no conjugation was seen and on agar the swimmers failed to develop. The temperature of the water was high and this may have explained the failure to conjugate.

Subsequently (December 1940) agar preparations of both Cambridge and Grahamstown material proved very successful in yielding data as to gamete formation and behaviour. In both cases nets from old cultures (i.e. cultures which through crowding had been slow in developing) were used, the nets having been separated out and transferred to fresh culture solution in larger vessels. When put on agar, some were found to have started net formation, others showed signs of imminent division. In several of the plate cultures many of the coenocytes produced swimmers: in a few cases nets were formed. In the pavement stage it was found possible to distinguish those coenocytes which were going to form gametes from those about to produce zooids by the larger diameter of the polygons in the latter case. Here in all the cases observed, formation and liberation of swimmers followed one or other of the methods described above, but nearly always the swimmers were liberated directly without formation of a vesicle. Nevertheless, they often showed a strong tendency to hang together about the point of exit in a large mass within which they could be seen moving with great vigour, beginning to conjugate immediately. In many cases separation was incomplete and clusters of two or more gametes attached to one another by fine protoplasmic strands escaped with the rest (Fig. 6. b). Some such incompletely separated gametes were watched struggling to free themselves, pulling in opposite directions and twisting round until the connecting strands were at last broken. Often conjugation took place actively within the partially emptied coenocytes.

(c) *The Vacuolar Membrane.*

In view of the rôle played by the vacuolar membrane in net formation, its behaviour during the formation of gametes was of special interest. The shrinkage observed in the mass of swimmers suggests a form of plasmolysis: the extreme density of the swarming mass of gametes usually makes observation of the membrane peculiarly difficult, but in a partially starved coenocyte with an extensively fenestrated protoplast the number of gametes formed is very much smaller and observation is less difficult, though even there by no means easy. Where contraction is very great it seems probable that the membrane may have collapsed completely, but sometimes at any rate it persists though greatly reduced in size and as

the coenocyte empties it can be clearly seen (Fig. 6, c, from a photograph). In some cases an optical section of the coenocyte showed two processions of gametes one on each side, moving along in single file one behind the other from the mass of as yet unseparated gametes at one end of the coenocyte to the ruptured end where liberation had commenced. Here the central vacuole was still large and the gametes were kept in the surface layer between its membrane and the outer wall. In such cases it seems as if liberation is in part at least regulated by pressure set up within the coenocyte and not simply a voluntary act on the part of the gametes and, if so, then it is probable that here too the rôle played by the vacuolar membrane is a significant one, if less so than in the formation of the daughter net. In those cases mentioned above where the central vacuole had been divided into a number of distinct parts each enclosed in a distinct membrane, if the coenocyte produced swimmers the stream of escaping swimmers carried along with it the separated parts of the vacuole, some spherical, some sausage shaped, sweeping them along and even carrying them out of the coenocyte into the water like a series of microscopic toy balloons. In this case the membranes seem to have been exceptionally persistent.

(d) *Form of the Gamete.*

The gametes are smaller than the net-forming zooids ($4-5\mu \times 5-7\mu$) with flagella a little more than body length inserted to one side of the clear anterior apex, which is sometimes drawn out into a small beak; near the points of insertion of the flagella one contractile vacuole is fairly easily seen, more rarely two, but as pointed out above there are probably always two with alternating periods of pulsation and therefore very difficult to detect. The nucleus lies rather to one side, causing a slight bulge in the clear end so that the gamete, like the zoospore, is slightly asymmetrical. The chloroplast varies; sometimes it appears to be a single oblique lobed structure containing a fairly distinct pyrenoid; more usually it is divided into a number of chloroplasts similar to but less distinct than those of the zooid. In these there is no pyrenoid, but starch is present in each chloroplast. There is usually a small slightly elongated eyespot which however is sometimes indistinguishable. Response to light varies; sometimes there is marked phototaxis but where the urge to conjugate is strong it apparently overcomes the influence of light (Fig. 6, a; Pl. XII e).

(e) *Conjugation.*

Swarming was sometimes prolonged, sometimes of very brief duration. In the last series of cultures described above, conjugation usually began immediately on or even before liberation: in the mass of swimmers hanging

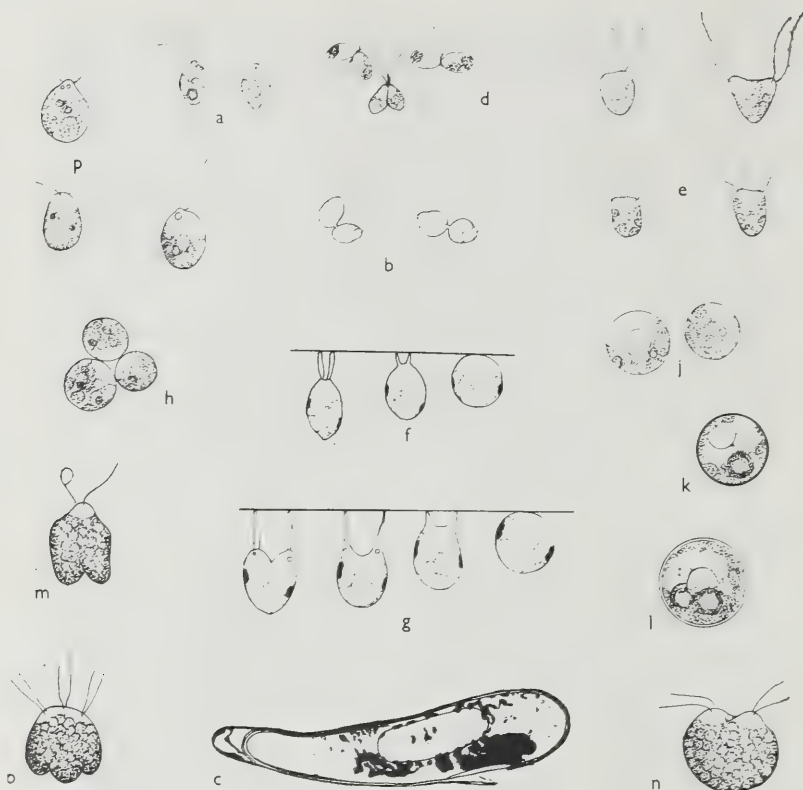


FIG. 6.—*H. reticulatum*. Sexual Reproduction. a, gametes; b, incomplete separation; d, conjugation; e, abnormal fusions probably resulting from incomplete cleavage; f, g, two planozygotes coming to rest and rounding off; h, newly formed resting spores; and j, enlargement just beginning; k, l, developing zygospores; m—o, composite zoospores; p, second type of swarmer; c, coenocyte in process of swarmer formation, majority of swarmer already escaped, vacuolar membrane (vm) still intact but much contracted. From a photograph.

c $\times 25$; b, d, m—o $\times 500$; e $\times 800$; other figures $\times 1000$.

together in dense clumps round the place of exit, associated pairs were seen almost at once and in a few seconds planozygotes were easily distinguished by their greater size, even before the clumps dispersed. Later, as the coenocyte gradually emptied, conjugation could be seen taking place actually within the coenocyte.

Association of gametes starts at the apex, the conjugating gametes coming together at an angle and rotating together in a most characteristic

manner; less often "head on" conjugation was seen, but even here a little later the gametes are almost side by side. Fusion begins at the apex, extending backwards until the gametes are completely united.

Not infrequently cases were seen where active quadriflagellate bodies showed two distinct apices (Fig. 6, e). This may have been due to a different method of conjugation between free gametes but is much more likely to have resulted from incomplete separation such as has been described above, where pairs of incompletely separated gametes have fused to form a planozygote instead of separating completely. In a culture of Grahamstown material, swarming on the 27th August 1939, incompletely separated gametes were observed and such quadriflagellate structures (Fig. 6, b, e) were also present. Although completion of fusion in such structures has not been watched, the occurrence of both the two-headed quadriflagellate individuals and cases of incomplete separation in the same material seems significant.

2. The Zygote.

(a) *Active Phase, the Planozygote.*

On completion of conjugation the quadriflagellate planozygote is at first very active, almost if not quite as rapid in its movements as the gamete, hence to see detail of structure it was necessary to use iodine. Towards the end of the period of motility, however, movement slows down and with the immersion lens it is possible to see a considerable amount of detail, especially when gametes and planozygotes are mounted in a small hanging drop, as they tend to move towards the edge of the drop. The flagella can then sometimes be distinguished while still active; in iodine they, of course, show up more clearly. In many cases the two flagella-bearing apices coincide and all four flagella spring almost from the same point, but in many cases the two pairs of flagella remain distinct, separated by a colourless zone. At what stage nuclear fusion takes place was not determined. Many planozygotes were watched coming to rest, and as movement slowed down their structure was more clearly seen, the vacuolar apparatus in particular becoming much clearer. In several cases, where the two pairs of flagella were separate, a single vacuole could be seen at the base of each pair and between these two other vacuoles lying rather behind one another.

(b) *Transition to Resting Phase.*

After a varying length of time, sometimes only a few minutes, the planozygotes come to rest, tending to collect on any available surface—the coenocyte wall, other zygotes, air bubbles, etc. In nature the chosen

object is no doubt often a particle of soil, plant detritus, and so on, or even the surface film of water. Often they are heaped up against one another, forming small masses of resting spores (Fig. 6, h).

It was interesting to watch planozygotes coming to rest and in several cases the process followed the following sequence:—The planozygote attached itself to some surface by the tips of the fully extended flagella, in which for a time undulating movements could be seen; then in those cases where two apices could be distinguished the two flagella of each pair appeared to fuse, the apices being somewhat drawn out. The flagella then thicken and shorten and are finally completely retracted into the protoplast, in the process the body of the zygote being gradually pulled up against the surface to which the flagella had adhered. The shape of the zygote has meanwhile altered, becoming first ovoid then finally more or less spherical: if there are many together they tend to flatten where they are in contact. The vacuoles continue to pulsate for a second or two longer, then they too disappear. Occasionally, when the tips of the flagella have become attached to the substratum, small bead-like parts are thrown off; more usually absorption is complete. As rounding off is achieved the eyespots, though small, often show up clearly. Most often the position in rest is such that the two eyespots lie on the circumference of optical section, sometimes close together, sometimes some distance apart. They are smaller and much less distinct than in *H. patenaeforme*. The whole process of rounding off is completed within a few minutes of the coming to rest of the planozygote (Fig. 6, f, g).

(c) *Formation of the Zygosporc.*

In the course of the next few hours, a wall is secreted, the chloroplasts merge into one and a pyrenoid appears. In some cultures, where the conjugating gametes had an undivided chloroplast with a pyrenoid, the young zygosporc showed two more or less distinct pyrenoids. The presence of two pyrenoids in the young hypnosporc would be a useful criterion by which to determine its zygotie constitution were this a constant feature. Unfortunately this is not the case, since many hypnosporcs which subsequently behave as zygosporcs possess only one pyrenoid even just before germination. On the other hand, in vigorously growing hypnosporcs, whether zygotie or azygotie, several pyrenoids may develop even when there was only one to begin with (Fig. 6, j—l).

C. EXPERIMENTS AND CULTURES.

(a) *Second Type of Swarmer.*

In rather old cultures of both the Cambridge and Grahamstown strains of *H. reticulatum* flocculent masses, often adhering to the coeno-

cytes, were common, particularly among daughter-forming nets. These were found to be largely composed of rounded cells apparently identical with the hypnospores already observed in *H. patenaeforme* and put on agar they developed similarly. Hence experiments were started to determine, if possible, the nature and origin of these spores.

Attempts were made to induce gamete formation by the methods described by Klebs (1896, p. 141, et seq.). Cultures in various sugar solutions, 0.5 per cent Maltose, Glucose and Sucrose respectively, were made, using parts of one and the same net, a large and healthy one not yet quite mature. Small beakers were used, each half filled with the solution and inoculated with a small portion of the net, the portions selected being as closely comparable in size and stage of development reached as possible. Two cultures in each solution were made, one put in the light, the other in the dark for 24 hours. No positive results were obtained.

The cultures were then kept in the light for a fortnight during which no form of reproduction was seen although in the control experiment, using normal culture solution, daughter nets had been formed. Many of the coenocytes in the sugar solutions were dying, but on transference to culture solution the surviving coenocytes recovered, continued development and in every case in most of the coenocytes daughter-net formation took place, while here and there a single coenocyte liberated swarms.

In other experiments in a number of single-net cultures in rather small beakers, it was found that the net began to form daughter nets and that then the process stopped, leaving about half the coenocytes intact. Later a number of these coenocytes became reproductive but instead of forming daughter nets, the contents escaped as a mass of active swarms.

A series of experiments was then started, putting fragments of mature nets of both strains on agar in a drop of culture solution. Mature coenocytes were chosen, preferably those that were showing signs of approaching division, i.e. becoming congested in appearance or even entering the pavement stage. Daughter nets develop surprisingly well under these conditions; nets are formed normally and develop rapidly, the only difference being that as it grows the cylindrical net lies flat on the agar since there is not sufficient free liquid to float it out, and for the same reason as the net elongates it is thrown into folds. Transferred to culture solution from the agar, the nets immediately assume their normal form. If the net used for the experiment had already begun daughter-net formation before the fragment was detached, the majority of the coenocytes on agar also formed daughter nets, as a rule in a very short time, often by the following day; some 24 hours later most of the remaining coeno-

cytes were usually liberating swarmers. Occasionally this order was reversed, many of the coenocytes on agar liberated swarmers, then after a period of quiescence the remainder began to form daughter nets. These sequences happened repeatedly both on agar and in culture fluid. In practically every case each culture yielded both daughter nets and swarmers from the same parent net.

In yet another experiment, three 4-inch nets (Grahamstown strain) were isolated in three large closed tubes of culture solution. Two days later, two of the tubes were green with swarmers. In the third tube no swarmers were formed but later the net produced daughter nets.

The two strains behaved somewhat differently in respect to swarmer formation, the Grahamstown material tending to form swarmers more readily than that from Cambridge. This cannot, however, be regarded as a fundamental difference between the two; more probably it is only a function of previous environmental conditions, whether free or in culture.

In all these experiments, mostly carried out early in the season (late winter or spring), conjugation was rarely observed among the liberated swarmers; nevertheless, it was at first assumed that they were normal gametes. Repeated examination with the immersion lens, including careful measurements of large numbers, however, showed that they were identical in size and structure with net-forming zooids, except that, being unrestricted, movement was much more active, and correlated with this, the flagella better developed and appreciably longer. The process of formation was identical with that of the net-forming zooids, complete division of the chloroplast of the coenocyte was followed by the appearance of the polygonal areas of the pavement stage, these being of the same size as in net formation (i.e. $7-9\mu$ as against $4-6\mu$ in gamete formation). But instead of contact between neighbouring zooids being re-established and movement ceasing, the activity of the motile bodies grew progressively stronger and finally the mass of active zooids moved unrestricted in the coenocyte, the vacuolar membrane apparently having disappeared. Finally, the wall ruptured and the zooids swarmed out into the water much as do the gametes. Each liberated zooid (Fig. 6, p) was ovoid in shape with oblique clear apex containing the nucleus and bearing two flagella, rather more than body length, inserted slightly to one side. Near the insertion of the flagella one or two contractile vacuoles could sometimes be seen; there were several clear green chloroplasts, the number of which varied from three or four large to six or more proportionately smaller. No pyrenoids were present but each chloroplast contained starch. Hyaline globules were often present between the chloroplasts. A small eyespot could occasionally be distinguished but more often no trace of one could be seen and there was a marked absence of

phototactic response; these swimmers, instead of congregating on the side of strongest illumination tended to collect on any solid object, such as the walls of coenocytes, whether empty or still living. Occasionally, however, well-marked phototaxy was exhibited, in which case an eyespot was fairly conspicuous. Gelatinization of the wall does not proceed so far in the formation of such zooids as in net formation; the outer membrane suffers little distortion and after liberation of the contents the empty coenocytes may persist for some time. If swarming, whether of true gametes or these larger zooids, has been general the empty walls are often thickly covered with hypnospores, in fact occasionally large portions of such an old net persisted outlined in a film of developing spores. On agar the zooids tend to collect round the edge of the drop of free water in which they lie irrespective of the direction of light. In general, such zooids form hypnospores without conjugating but occasionally apparently conjugation may occur.

A few specific cultures with their results are described briefly here as they serve to indicate the range in behaviour of swimmers in this species.

(b) *Cultures.*

(1) 22 August 1939. Part of a large net (third asexually produced generation of Miss Britten's Grahamstown material) was transferred from culture solution to distilled water. Almost immediately it began to liberate swimmers. These were rather large ($5.5 \times 7\mu$ to $7 \times 9\mu$) with rounded poles, each with numerous clear green chloroplasts rich in starch, with globules of reserve material, no pyrenoid and no distinguishable stigma. The slightly oblique clear anterior pole contained several moving granules and bore two delicate flagella inserted separately (Fig. 6, p). They soon settled down and rounded off to form hypnospores. No conjugation was seen and in samples fixed in iodine no quadriflagellate individuals could be found.

(2) 27 August 1939. Again Grahamstown material. The water in the cultures was found to be green with swimmers. The majority of those examined were biflagellate but some quadriflagellate individuals were present. Here the chloroplasts were much less distinct, the swimmers had more the appearance characteristic of *H. patenaeforme* and on the whole the size was rather smaller than in Culture 1, although some were larger, possibly planozygotes ($4 \times 7\mu$ to $6 \times 11\mu$). Most of the quadriflagellate bodies however did not look like normal planozygotes; their appearance suggested incomplete separation rather than conjugation.

(3) 3 September 1939. In a liquid culture of Cambridge material vast numbers of swimmers were liberated. These were first observed at 9 a.m. and movement continued for many hours. Of samples put on agar, many were still active at 11.30 p.m., while a few were still moving at noon the following day. No eyespot could be distinguished but there was some response to light, in drops put on agar some collecting on the side of the drop nearest the light. There was a marked tendency to form clumps and many were seen associated in pairs though actual fusion was not seen. There was considerable variation in size; the smallest measured $4 \times 6\mu$, the largest $6.5 \times 9\mu$, with body length flagella.

(4) 30 October 1939. Cambridge strain. Fragments of a coenobium which had begun daughter-net formation were put on agar in drops of culture solution. By noon the following day many of the coenocytes were liberating swimmers. These were large ($7 \times 9\mu$ to $9 \times 12\mu$), some with a few large discoid chloroplasts, some with smaller ones. A small but distinct eyespot could be seen and sometimes a single contractile vacuole. A few coenocytes did not liberate swimmers but the following day formed daughter nets. In many similar experiments as a rule daughter-net formation occurred first to be followed a day or two later by liberation of swimmers.

D. THE HYPNOSPORE.

(a) *Development and Germination.*

As in *H. patenaeforme* the small newly formed resting spores of *H. reticulatum* develop quickly on agar, particularly in warm spring weather, development following the same stages as in that species, with minor variations in detail. The chloroplast may become extensively vacuolated; usually the initial number of pyrenoids (either one or two) persists but sometimes more appear (Fig. 6, j—l; Plate XIX, A—C). Under the most favourable conditions growth is rapid and germ nets were obtained 12 days after the liberation of the swimmers; for example, in September 1939 swimmers liberated on agar on the 8th and transferred to fresh agar plates were ready to germinate (13—40 μ diameter) by the 17th, when some were removed from the agar and put in a watch glass of culture solution. By the morning of the 20th, in addition to many polyhedra, germ nets were already present.

Transference from agar to dilute culture solution (about half strength) usually brought about germination in such young spores, that is, those which were well developed and still green but in which the chloroplast was beginning to darken and appear congested and granular. As in *H. patenaeforme* older spores, in which the colour had changed from green to golden brown and the walls had thickened considerably, usually required several changes in the concentration of the nutrient medium before germination could be induced, for example, transference to distilled water for 24 hours, sometimes less, then addition of culture solution. In

PLATE XIX. *Hydrodictyon reticulatum*.

C, Grahamstown, the rest Cambridge culture.

- A. Young hypnospores, some with two pyrenoids (1021). $\times 1100$.
- B. Three hypnospores and two coenocytes formed direct from swimmers(1019). $\times 1100$.
- C. Group of hypnospores, three germinating, and two zoospores just liberated from a germinating zygospore(971). $\times 150$.
- D. Compound polyhedron(978). $\times 375$. E. Another, composed of two polyhedra slightly attached, one degenerated, the other nearly mature(978). $\times 375$.
- F. Development of polyhedron first seen 3.10 p.m., photographed at 5.45 p.m., showing arrested development; vesicle wall thick, wall of polyhedron attached at one side(981). $\times 375$.
- G. "Basket" net in vesicle; group of three cells and a single cell formed by swimmers separated from the rest in net formation (980). $\times 250$.
- H. Polyhedron and germ net showing vacuolar membrane (slightly retouched) (1033). $\times 114$.
- J. Group of germ nets(1040). $\times 140$.
- K. Single particularly well-formed net in shape of a hollow sphere; round hole indicating position of vacuolar membrane bottom right(1041). $\times 150$.
- L. Germ nets printed dark to show membrane of vesicle; in upper net vesicle wall in form of the polyhedron(1039). $\times 140$.
- M. Germ nets, nearly mature; two, large hollow sacs, more or less isodiametric, the other two tangled masses(1036). $\times 17.5$.

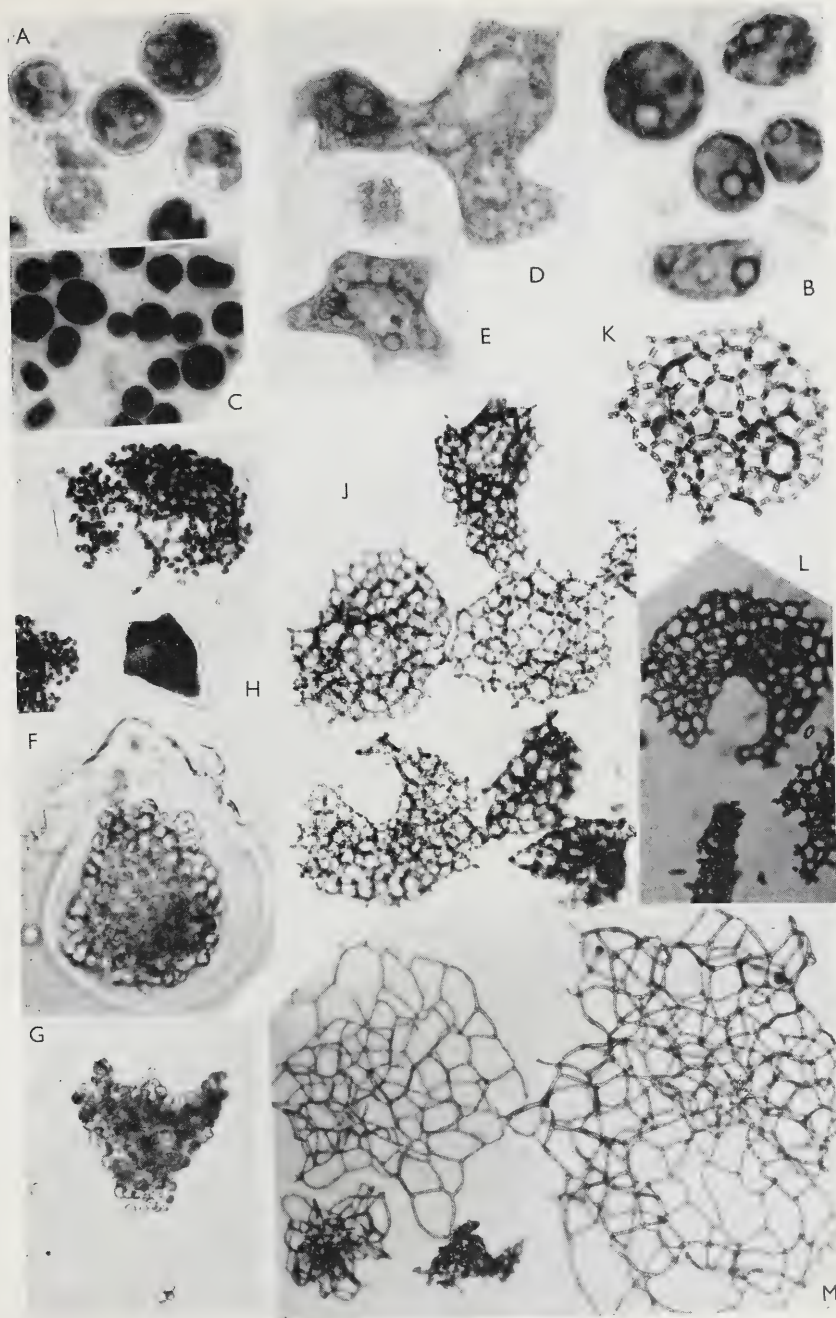


PLATE XIX. *Hydrodictyon reticulatum*.

some cases one such change sufficed, in others the double change had to be repeated several times before it was effective and then only some of the spores usually responded. Here, too, exposure to direct sunlight for a short time was also found to be effective at times in inducing germination.

(b) *Zygotes and Azygotes Among the Hypnospores.*

Just as in *H. patenaeforme* here, too, the hypnospores usually proved to be a mixture of zygotes and azygotes, the relative proportion varying in different cultures.

Early in the investigation germ nets from hypnospores of the Grahams-town strain were isolated; one of these germ nets formed some daughter nets and also liberated a number of swimmers. Some of the latter were put on agar and the resultant hypnospores in turn germinated; the proportion of zygospores was high, but nevertheless azygotic hypnospores were also present. In every case, whether the swimmers were zooid-like or gametic, the resultant hypnospores were found to be similar, that is, in all the cultures they included both zygotes and azygotes, the former predominating.

Other things being equal, here, too, the azygospores germinated earlier than the zygospores, each producing a single zoospore (Pl. XII, G) irrespective of size. Later in the day the zygospores commenced to germinate, the contents dividing before the vesicle was fully formed (Pl. XII, F). Germination was commonest by day, the azygospores usually germinating in the morning or early afternoon, the zygospores a little later, liberation of zoospores continuing into the night.

(c) *The Zoospore.*

If the germinating spores are green, the zoospores formed on germination are also green, a very dark intense green produced by numerous closely packed rounded chloroplasts and very well represented in Pringsheim's figures (1861, Fig. 9). With the immersion lens the separate chloroplasts show clearly. If, on the other hand, the germinating spores are golden or golden brown, some of the haematochrome survives in the zoospores which are consequently themselves brownish green or brown. In such zoospore either the haematochrome masks the chloroplasts or division of the chloroplast is incomplete and it is difficult to distinguish separate chloroplasts, the zoospores appearing coarsely granular.

The zoospore may be vigorous, showing both metabolic and flagellary action. The two delicate flagella are usually less than body length and tend to be directed backwards, consequently, since at the best of times they are difficult to distinguish, should they be in this position and lying close to the body of the zoospore, it may appear as if there were only

one flagellum present, as figured by Pringsheim (l.c., Fig. 9). In all such cases careful examination revealed the second flagellum bent back along the side of the zoospore and almost hidden by it (Pl. XII, G).

Few cases of very large zygospores producing more than four zoospores were obtained in this species. The great majority of zygospores regularly produced four zoospores on germination although there were nevertheless many exceptions.

(d) Compound Zoospores.

In some old spore cultures on germination a fairly high proportion exhibited incomplete cleavage, resulting in variously shaped zoospores showing two, three or even four flagella-bearing apices (Fig. 6, n, o). Here again such zoospores were usually less rapid in their movements than the normal type and the separate pairs of flagella rather more easily seen. Although composite in constitution, such zoospores settled down and formed a single polyhedron in the normal way, each, like the zoospore which formed it, multinucleate from its inception.

Occasionally, cases were seen where cytoplasmic cleavage was almost complete and two zoospores slightly attached to one another resulted: these moved together but on settling down formed two polyhedra joined to one another at one point. If the connection is fairly wide, as in Plate XIX, D, the two polyhedra may eventually merge in net formation; in this case the larger polyhedron probably represents three, the smaller the fourth of four incipient zoospores formed by a germinating zygospore. The second figure (Pl. XIX, E) shows two polyhedra attached at one place by the wall only, one having developed for a time and then degenerated.

Such composite zoospores may result directly from incomplete cytoplasmic cleavage after completion of nuclear division, or they may arise from partial or complete reunion of the zoospores after differentiation and before liberation. One such case was watched in detail. When first observed the germinating zygospore was nearing completion of vesicle formation, the protoplast passing out into the vesicle was tetrahedrally divided and flagella already being differentiated and becoming active: of the four zoospores two were elongated and lying superimposed on one another, while the remaining two were slightly larger, rounded and separate. As they were watched the two former coalesced and then the two latter, side by side; the two apices in each case united laterally and the two pairs of flagella continued to move with an undulatory movement. Unfortunately, before actual liberation took place the whole structure was crushed and hence the further fate of the two resultant composite swimmers could not be followed. No doubt this was a pathological case brought

about at least in part by pressure of the cover slip, though that alone could hardly have induced such complete union and yet allow continued flagellary action. It is nevertheless significant and serves to emphasize the fact that the zoospore, like the swarmer, is a naked mass of protoplasm and capable of uniting with its fellows. In this, as in other cases of composite zoospores, the fact that the various pairs of flagella continue to function separately and independently throughout suggests that there has been no nuclear fusion, the two nuclei remaining distinct in the zoospore. This was confirmed where similar zoospores were treated with iodine.

In some cultures the hypnospores regularly produced two zoospores. Careful observation showed that as the vesicle formed cleavage began; the first cleavage was followed by an incipient second cleavage which was not complete. Instead, the lines of the partial cleavage disappeared and two zoospores only were formed, apparently normal but actually binucleate and with two pairs of flagella (Pl. XII, F; Pl. XVIII, C). What was the cause of this peculiar behaviour in a culture which appeared to be quite normal and healthy could not be determined; in this species at times it seems to be of comparatively common occurrence.

E. THE POLYHEDRON AND GERM NET.

(a) *Formation of the Germ Net.*

The formation of the germ net in this species has been watched in a number of cases. Apparently the method of formation of the vesicle and the net itself differs slightly in several details from that in *H. patenaeforme* although in the main the process is the same in the two species. Firstly, in all the cases actually observed, the thin outer membrane of the wall was sloughed off, remaining as a crumpled ghost of the polyhedron adhering to one side of the vesicle which is formed from the inner wall layers only. But in several recently formed young nets, the vesicle, instead of being rounded, retained the form of the polyhedron (Pl. XIX, L), suggesting that in those cases the whole polyhedron wall, including the outer membrane, had contributed to the wall of the vesicle. The point is not of great importance since the later more extensive observations have shown that even in *H. patenaeforme* it is exceptional, and that in the majority of cases there, too, only the inner wall layers are involved in vesicle formation.

The second variation comprises the form of the net and, probably intimately connected with it, the longer persistence of the vacuolar membrane. As movement begins in the mass of zooids it often appears to be localized at one point where it is strongest, tending to bring about separa-

tion there so that the mass opens out like a basket (Pl. XIX, G) and may ultimately become flattened. But nets completely in one plane are rare and apparently occur only when there is a comparatively small number of zooids and then only occasionally. Where the number of zooids is larger and a flattened net is still formed, the marginal part only is single layered, the central part made of two or more layers usually joined to one another by cross pieces formed by zooids lying so as to join one plane with another. In such flattened or partially flattened nets the marginal cells may show horn-like processes as in *H. patenaeforme*.

In far the greatest number of cases, however, movement is evenly distributed throughout the mass of zooids which consequently move apart more or less evenly following the expanding vesicle and kept in this position by the vacuolar membrane. Thus a hollow sac, more or less spherical in form results, particularly well formed when the zooids are numerous. Often it is open to one side, the opening being approximately circular in shape (Pl. XIX, J, K). Sometimes the net is crescent shaped, sometimes a nearly complete sphere (Pl. XIX, K), but whatever the variations in shape, the net is most often in the form of a hollow sac, as emphasized by Pringsheim and figured by him (Pringsheim, l.c., Fig. 19).

As indicated above, the vacuolar membrane here plays a most important rôle in regulating the early movements of the mass of zooids and determining the form of the net. Normally, at the beginning of net formation it occupies the centre of the polyhedron so that from the beginning, as in daughter-net formation, the zooids form a single layer between it and the wall. As the latter expands in the process of vesicle formation the zooids move outwards, still kept in place by the slightly enlarging vacuole. Enlargement of the vesicle, however, proceeds more rapidly than does that of the vacuole and its membrane so that the space between the latter and the wall of the vesicle becomes much greater, allowing greater freedom of movement to the zooids and thus allowing for more variation in the form of the germ net. When control is most complete and union between the zooids takes place early in the process, the sub-spherical form of sac results, and in the majority of such nets, as also in the basket type, the vacuolar membrane persists to a late stage and it is often possible to distinguish it as a small spherical bubble, lying to one side of the vesicle often adjacent to the circular space in the net referred to above, even after the completely formed net has begun to enlarge. Unfortunately, in the prints here reproduced it shows in only one (Pl. XIX, H) and not too clearly in that. Both membranes are so delicate in proportion to the net itself that it is difficult to obtain prints which show both the vesicle wall and vacuolar membrane as well as the

germ net itself, except in dark prints such as Fig. L on the same plate. All the nets figured on this plate (except those in Fig. M) were actually still enclosed in the vesicle and in several the vacuolar membrane was still intact.

Yet another type of net is frequently met with, particularly in old crowded or contaminated cultures. Here apparently the vacuolar membrane has collapsed or disappeared at an early stage while at the same time the vesicle has developed imperfectly. As a consequence the zooids have not separated normally but have united irregularly, the result being a formless tangled mass instead of a well-shaped net (Pl. XIX, M, two lower nets on the left). Yet even such nets may develop and give rise to a population of normal nets; the larger of the two tangled nets in this photograph shows some at least of the constituent coenocytes developing well. Found occasionally in cultures of *H. patenaeforme*, they were much commoner in *H. reticulatum* cultures, but since there are strong indications that they are the result of unfavourable conditions, too much stress must not be laid on them.

(b) *Form of the Germ Net.*

Many hundreds of germ nets of *H. reticulatum* have been raised in culture and counts made from time to time confirm the impression first obtained that the hollow sac is the typical form of the net in this species, as already recorded by Pringsheim (1860), while in the two other species the flat net predominates. The results of two such counts are given to illustrate the results obtained:—

1. In October 1939 a count of some 200 germ nets of the Grahams-town material gave the following results:—

Nets formed entirely in one plane	..	..	..	0
Nets several layered in centre, margin single layered	..			25%
Nets more or less spherical, usually a hollow sac, occasionally a tangled mass	..	..	..	75%

2. In June 1940, again in Grahamstown progeny, a few perfectly flat nets were obtained and counts gave the following results:—

Nets formed entirely in one plane	..	..	..	20%
Nets several layered in centre, margin one layered	..			35%
Nets a hollow sac or a tangled mass	..	..	..	45%

This culture gave the highest percentage of flat nets of any culture. As a whole, the Cambridge strain showed a slightly stronger tendency to the formation of flat nets than that from Grahamstown, but even there, nets entirely in one plane were rare and always comparatively few celled

The number of coenocytes per net varies considerably depending on the size of the polyhedron. In one perfectly flat net 245 coenocytes were counted, probably representing a true number of 256 zooids. In these cultures nets with fewer coenocytes were rare; the flat net figured by Mainx (1931, Fig. 17) consists of 128 coenocytes, but such a low number is apparently unusual when conditions are favourable. Most of the germ nets raised during this work were sac-shaped and consisted of considerably more coenocytes but rarely as many as in the two other species, where the most usual numbers are 512 and 1,024.

(c) *Duration of the Germ Net.*

Germ nets raised in spore cultures have been kept in culture side by side with those of *H. patenaeforme*. Even when isolated the germ net of *H. reticulatum* never reaches the size of those of the former species and twisting of the coenocytes though occasionally observed is unusual. Whereas the germ nets of *H. patenaeforme* enlarge greatly and persist for a long time forming the mature nets, which eventually form swarmers those of *H. reticulatum* reach maturity after a very much shorter vegetative period (Pl. XIX. M), are much smaller even when fully grown and nearly always proceed at once to daughter-net formation. Thus the germ net very soon gives rise to a population of closed cylindrical nets of the traditional form on which the diagnosis of the genus was originally based. It is true that here and there germ nets liberating swarmers were observed, but even then it was only a few of the coenocytes which behaved thus, the majority forming daughter nets.

The germ net consists of few coenocytes, at most a few hundreds as compared with several thousands in the vegetatively formed daughter net, but owing to this very fact its coenocytes have more room to develop and are usually well formed and equal in size to those of the larger nets. Therefore the daughter nets it produces, though few in number, are typically large and well formed, consisting of the large number of coenocytes found in all well-developed asexually formed nets regarded as characteristic of the species. Hence, even in the second generation, the progeny of a single germ net may consist of thousands of large cylindrical nets. Bearing in mind therefore the small size and comparatively brief duration of the germ net, it is hardly surprising that all descriptions of *H. reticulatum* have been based entirely on the nets formed in asexual reproduction and the germ net entirely ignored.

F. COENOCYTES FORMED DIRECT FROM MOTILE CELLS.

Here, as in *H. patenaeforme*, it is found that swarmers, of whatever kind, instead of passing into the resting stage and forming hypnospores

may develop directly into a coenocyte and also that net-forming zooids may on occasion develop independently into either coenocytes or hypnospores.

(a) *Coenocytes from Swarms.*

On an agar plate of *H. reticulatum* from Grahamstown, in which liberation of swarms had resulted in the formation of hypnospores among the latter were found a number of young coenocytes. On the 8th September a number of such isolated coenocytes of various shapes, some spherical, some elongated (Pl. XIX, B), were isolated in a jar of culture solution. Here they developed perfectly normally, except that while enlarging they retained their various shapes, and eventually each produced a daughter net. The result was an amusing collection of nets of various shapes, some spherical, others ellipsoidal and yet others of quite complicated shapes according to those of the young coenocytes; a few were even cylindrical. On the 16th November these nets were transferred to fresh culture solution and four days later had produced a mass of daughter nets of the typical cylindrical form.

In this case the coenocytes were formed by swarms which had escaped in the usual way and after a period of active movement had settled down on the agar. The majority rounded off, secreted a wall and formed hypnospores which, in turn, after a period of growth either germinated at once or entered a more or less prolonged period of rest according to existing conditions. In a small proportion, however, although a wall was formed, it did not thicken and vegetative development continued without an intervening period of quiescence; nuclei and pyrenoids multiplied, the whole cell enlarged, assuming various shapes in the process, the chloroplast soon attained the form characteristic of the developing coenocyte in a net, except that, in general, its shape was different; when maturity was reached each proceeded to form a daughter net. The growth impulse was stronger than in those which formed hypnospores, overcoming the impulse to form a resting spore.

(b) *Coenocytes and Hypnospores from Zooids.*

As already noted, the same thing may happen in the case of net-forming zooids, whether in daughter nets or germ nets, which for some reason have failed to make contact with their fellows. Occasionally a few such are seen inside a coenocyte which has formed a daughter net; whether such zooids continued development was not established, but in the light of other observations seem probable. These were made from

time to time in cases where the wall of a net-forming coenocyte had been torn while net formation was actually in progress, resulting in the escape of some of the zooids: on agar it was possible to note the behaviour of such isolated zooids and it was found that they might develop into either coenocytes or hyphospores according to the stage reached at the time of their liberation.

(c) *Coenocytes from Zoospores.*

Very occasionally zoospores liberated in germination of hyphospores were found to develop into coenocytes instead of polyhedra. Here the polyhedral growth-phase was cut out and the coenocyte is not far removed from a polyhedron in its nature and, as in *H. patenaeforme*, may show incipient conical outgrowths of the wall. It is however considerably greater in size and when mature usually forms a daughter net. It is noteworthy that all such coenocytes, whatever their origin, "run true to form", according to the species, i.e. in *H. patenaeforme* they eventually produce swarmers, in *H. reticulatum* usually a daughter net, only very occasionally swarmers.

(d) *Net Formation Outside the Vesicle.*

Occasionally in nets, which had been torn open during net formation, some of the zooids continued to unite with one another although separated from the bulk of their fellows, small fragments of nets forming free in the water: this only occurred if net formation was already far advanced, otherwise the separated zooids behaved as in case (b), forming either resting spores or coenocytes.

In the case of germ nets, it not infrequently happened that the vesicle was ruptured before net formation had been completed: this was particularly noticeable in nets of the tangled type where the vacuolar membrane had also been ruptured. As a result the zooids were imperfectly united and single cells or small groups resulted. Occasionally, if net formation were already far advanced, the net-forming urge was strong enough to overcome to some extent the disturbance caused by direct exposure to the surrounding liquid and the process continued on the floor of the watch glass in which germ-net formation was taking place, the unrestricted zooids continuing to unite, but, erratically, forming flat irregular nets or portions of nets. Union was generally only partial, some of the zooids remaining isolated. Nevertheless, both isolated zooids and fragmentary nets continued to develop, eventually forming either daughter nets or swarmers.

III. HYDRODICTYON AFRICANUM.

A. HISTORY AND DISTRIBUTION OF THE SPECIES.

In many ways the most striking of the species of *Hydrodictyon*, *H. africanum* is by far the most restricted in distribution. It had been noted on the Cape Flats certainly prior to 1913, since in the brief account of South African algae included in Marloth's *Flora of South Africa* (1913, Vol. I, Plate 2, Fig. 1) Mr. W. T. Saxton states that "the water net", *Hydrodictyon reticulatum*, is found in vleis in the spring" (p. 8), but the figure is undoubtedly of this species and not of *H. reticulatum*, as was erroneously stated. Its specific rank, however, was first recognized in America where it appeared in cultures of soil from the Cape Flats made in the Botany Department of the University of Chicago. There it was described and named by Yamanouchi (1913, p. 76) who also studied its development and life history as it appeared in culture.

The type locality from which Yamanouchi's material originated was Valkenberg Vlei, between Observatory Road and Mowbray. Large stretches of flat land lying between the Black River and the ridge on which Valkenberg Hospital and the Royal Observatory are built were formerly regularly inundated during the winter. Canalization of the river and extensive sewerage works have materially altered the whole area and *H. africanum* seems to have disappeared from this locality. Formerly, since the vlei was large and the comparatively shallow water seldom disturbed, it was possible to wade far out and find nearly perfect nets, some 12 inches or so in diameter, composed of clear translucent green beads, lying on the floor of the vlei in 9—12 inches of water, often among decorticated remains of plants of *Arthrocnemum natalensis* which survived in the water of the vlei; when dry much of the area was covered with this and other halophytic flowering plants.

The species has been found in a number of other vleis or pools near the Cape Peninsula, mostly in the north-western part of the Cape Flats; of these the chief are a number of small vleis on both sides of the Klipfontein Road, Mowbray (many of these too now non-existent, since it is a district that is rapidly being built over), an extensive shallow vlei near the Albion Brick and Tile factory on the road connecting the Klipfontein Road and Lansdowne Road, and Isoetes Vlei*, on the latter road, a little south of St. Patrick's Tower. Further to the south, it has occasionally been found in Groen Vlei (Pocock, December 1929), but has not been seen there for many years, whereas *H. patenaeforme* is abundant in this and in many vleis and ditches to the south and west in the Ottery district.

* Now, thanks to the initiative of Miss E. L. Stephens, a small nature reserve.

Passing northwards out of the Cape Flats region. *H. africanum* has been collected in Riet Vlei, beyond Milnerton, and at a few other stations on the coastal strip of country which is continuous with the Cape Flats and extends for many miles north of Cape Town. The chief of these are a vlei on the farm Nooienfontein (Mr. Brink's), two miles from Kuils River (Duthie, 18th September 1939), and Bokram (or Bokkeram) Vlei and other vleis or pans on the farm La Rochelle near St. Helena Bay, nearly a hundred miles north of Cape Town (Stephens, September 1932 October 1933). The last records are significant since it seems probable that further search might result in finding the species in many other temporary vleis in this region, still comparatively untouched by building operations.

All the localities on the Cape Flats where it has been found and a great part of the coastal strip are characterized by deposits of blown sand with a high salt and lime content from sea shells, etc., and the vleis, though when first formed approximately neutral, as the season advances become strongly alkaline with a very high pH concentration, quite unlike the vleis where *H. patenaeforme* abounds. Associated with *H. africanum*, in addition to the *Arthrocnemum* mentioned above, the following plants are noteworthy: among flowering plants a species of *Statice*, semi-aquatic species of *Crassula*, *Cotula coronopifolia*, and various species of *Juncus* and *Cyperaceae*, while among aquatic non-flowering plants the most outstanding are *Isoetes capensis* and *I. stellenbosiensis*, *Riella purpureospora*, *Nitella praeclara* and other Charophytes.

B. SPECIFIC CHARACTERS.

(a) Form of the Net and Coenocyte.

As in *Hydrodictyon patenaeforme*, the net in *H. africanum* takes the form of a flat plate or shallow saucer and since asexual reproduction by

PLATE XX. *Hydrodictyon africanum*.

Nets raised from spores and grown in culture at Rhodes University. F and G on agar.

- A. Developing hypnosporos (zygotic)(973). $\times 800$.
- B. Hypnosporos, higher focus, showing large vacuoles and several pyrenoids (972). $\times 800$.
- C. Nearly perfect young net, 512 coenocytes(974). $\times 114$.
- D. Young net with double centre(000). $\times 50$.
- E. Older net, a few coenocytes swelling up irregularly, majority still cylindrical (982). $\times 174$.
- F. Hypnosporos (large and small), some forming nets, on agar(975). $\times 27$.
- G. Net (somewhat crumpled) and spores, on agar(976). $\times 255$.
- H. Double-layered net showing uneven swelling of coenocytes. $\times 1.5$ approx.
- J. Large well-formed net, part of centre two layered, isolated when very young in a large dish of culture solution and left undisturbed; coenocytes (over 1,500) well rounded, beginning to separate, becoming spherical as they do so. $\times 0.3$ approx.

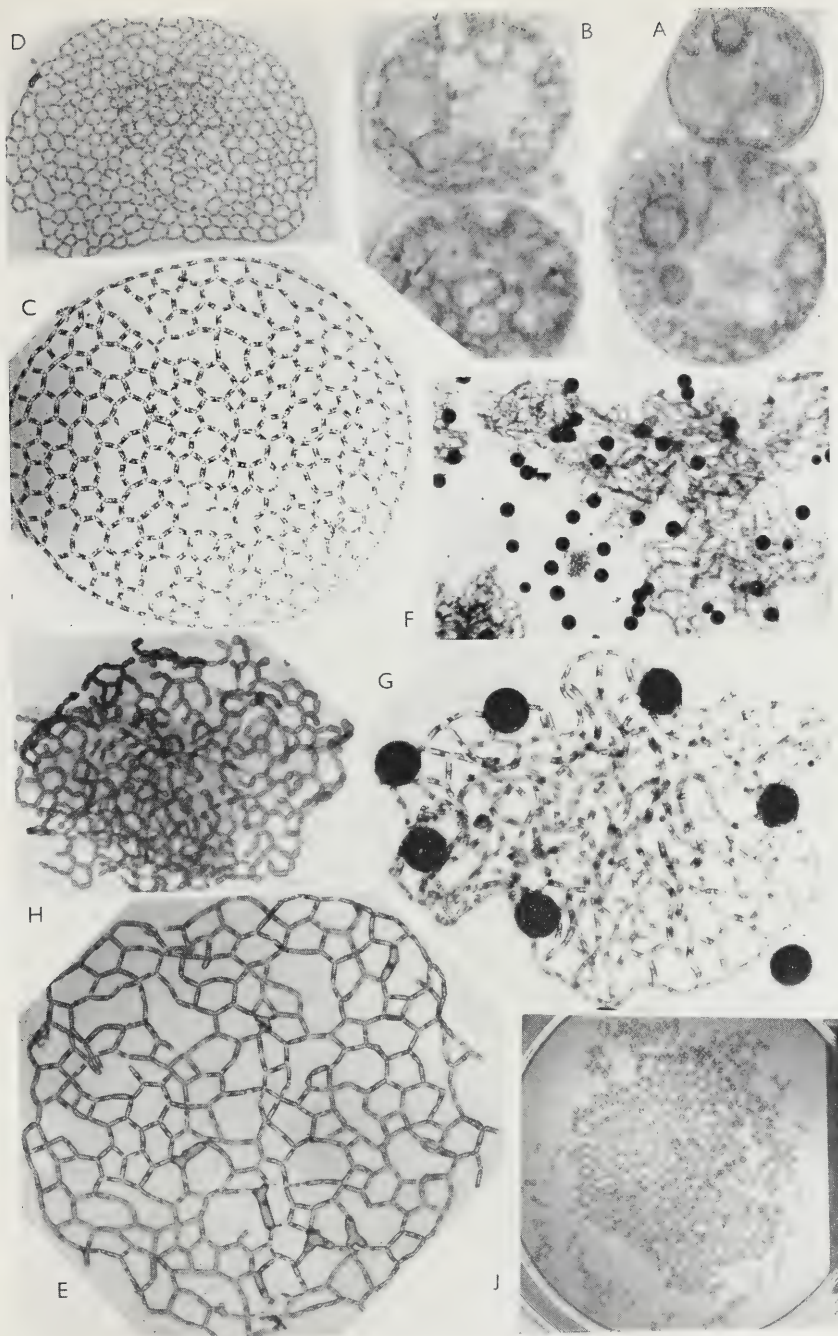


PLATE XX. *Hydrodictyon africanum*.

the formation of daughter nets is unknown, there is only one net in the life cycle. Further, the twisting of the coenocytes as they enlarge, so characteristic of the former species, does not occur in *H. africanum*, while the tendency of mature coenocytes to become dissociated noted in *H. patenaeforme* has become a regular phenomenon in *H. africanum*. Both of these characters follow as a natural consequence of the most striking feature which distinguished it from all other species of *Hydrodictyon*, that is, the form of the adult coenocyte. In the other species the coenocyte of the net retains the cylindrical form acquired at an early stage in the developing net; in *H. africanum* as the germ net develops the coenocytes become cylindrical, just as they do in the other two species, but very soon they begin to swell up, becoming at first barrel shaped and eventually almost spherical. As the form approximates to that of a sphere, the area of contact between adjacent coenocytes becomes progressively smaller in proportion to the whole surface and cohesion between the coenocytes grows gradually weaker until a light touch, or even agitation of the surrounding water, may serve to cause separation and the green network of rounded beads breaks up more or less completely into its constituent parts. Once freed, the coenocyte becomes completely spherical, the slight flattening at the surfaces of contact disappearing completely (Pl. XX, C, H, J; Fig. 8, j—l).

The age at which this dissociation takes place varies; it depends on a number of factors but normally it is never until the coenocytes have become nearly spherical, whatever their size. After separation growth continues in the individual coenocytes until they may reach a diameter of 1 cm. or even more. In nature such separate coenocytes tend to collect in hollows on the floor of the vlei like large translucent green marbles. This is well seen at Isoetes Vlei where there is a slight but perceptible current in the water between the two sections into which the road divides the vlei, and masses of coenocytes, varying from a millimetre to over a centimetre in diameter, collect in the deeper parts. Very slight agitation of the water brings them floating up to the surface whence they gradually sink once more, the specific gravity of the balls being very little greater than that of water. In this condition they are known to the local inhabitants of the Flats as "green frogs' eggs". If they are resting in deep water or in shaded parts of the vlei the colour is a clear bright green, no matter what the age; if exposed to strong sunlight, as in the shallower water, or in hot weather, a change of chlorophyll to haematochrome takes place and they become pale gold or reddish yellow in colour. If they collect in large masses in deepish water the colour change may take place in the upper layers, while lower down in the mass the coenocytes still retain their pristine green colour, and at the bottom, nets and parts

of nets with green coenocytes of various sizes not yet separated may be found. The colour change is not a function of age as suggested by Yamanouchi (l.c., p. 78) but is definitely a starvation phenomenon whether induced by too strong insolation or by deficiency in nutrient salts. In culture, such golden coenocytes may be induced to resume their green colour by addition of fresh culture solution, while removal to less strong light also helps. Where the colour change has resulted from actual food deficiency it is usually accompanied by extensive vacuolation of the chloroplast (Fig. 7. d). On the other hand, large coenocytes have been kept in a deep glass trough in shade in the Botany Department of the University of Cape Town for several months, remaining a deep green throughout.

(b) Duration of the Net.

The duration of the net depends very greatly on external conditions, primarily on rainfall, since the vleis concerned are all temporary. Temperature and sunlight are all of importance in determining not only the life of the vlei but also the rate at which the alga develops. In some seasons it is rare and quickly disappears; in others it may persist for weeks or even months. In windy weather, when the water of the vleis may be considerably agitated, the nets tend to break up into their constituent coenocytes more quickly than in calm weather. The life of the net as a complete plate is relatively short: it is usually in the form of separate coenocytes that it survives. If kept undisturbed they may remain unaltered for many weeks; disturbance or addition of fresh culture solution usually hastens disappearance. This is usually due to the formation of reproductive bodies but may be due to other causes. Certainly large coenocytes may produce swarmers, but swarmer formation may occur in quite small coenocytes, and for convenience of observation it is the latter that have been used in most of the present series of experiments.

C. STRUCTURE OF THE COENOCYTE.

In its essential features the structure is the same as in the other species, with slight modifications rather difficult to define and obviously correlated with the differences in size and shape, but consisting mainly in a thicker and tougher outer membrane and a somewhat coarser "grain" in the chloroplast.

(a) The Chloroplast.

The peripheral chloroplast is again a fenestrated sheet, but usually the fenestrations are rather larger and more noticeable than in the other

species, particularly *H. reticulatum*. Under conditions of starvation the fenestrations enlarge, merge into one another and the whole protoplast may be broken up into an attenuated network of lightly pigmented, slightly yellowish strands in which the pyrenoids are conspicuous (Fig. 7, d). On addition of fresh culture solution the chloroplast begins to extend in all directions, the vacuoles or fenestrations filling up more or less completely and the colour becoming green once more. Details of a culture illustrating these changes are given later.

(b) "Starved" Nets.

In some cultures where young partially starved nets have subsequently been transferred to abundance of fresh culture solution, coenocytes have developed rapidly—swelling up unevenly, some parts remaining narrow, others increasing greatly in diameter. This may result in bizarre shapes—coenocytes may become dumb-bell-shaped, or may swell up in the middle, the ends remaining narrow, or may even form a row of bead-like structures connected by narrow necks (Fig. 7, a—c). This is comparable to the similar behaviour noticed in *H. reticulatum* and seems to be due to the wall becoming altered at certain points, so that when growth is resumed only parts of it are able to enlarge, the altered parts remaining narrow. These and many other curious shapes were common in a series of cultures made during the period November 1939 to February 1940. The source of the material was the Klipfontein Road vleis; swarming had taken place freely in the laboratory, spore cultures on agar developed well and in mid-January many of the germ nets, which had been formed on agar by germinating spores some days previously, were used for further cultures. They had however undergone a period of semi-starvation on the agar during early stages of development and on removal to better conditions developed erratically. In some cases such nets formed on agar and left for some time have become almost completely colourless and yet have recovered, become green again and continued to develop normally on transference to fresh culture solution.

D. REPRODUCTION.

1. The Coenocyte.

(a) Size at Maturity.

For the detailed study of the formation and development of reproductive stages, young nets, parts of nets or separate coenocytes were isolated either in culture solution or on agar plates. It had been thought that the coenocytes did not become reproductive until they had reached a large size. During the investigation it was found that this was quite a mis-

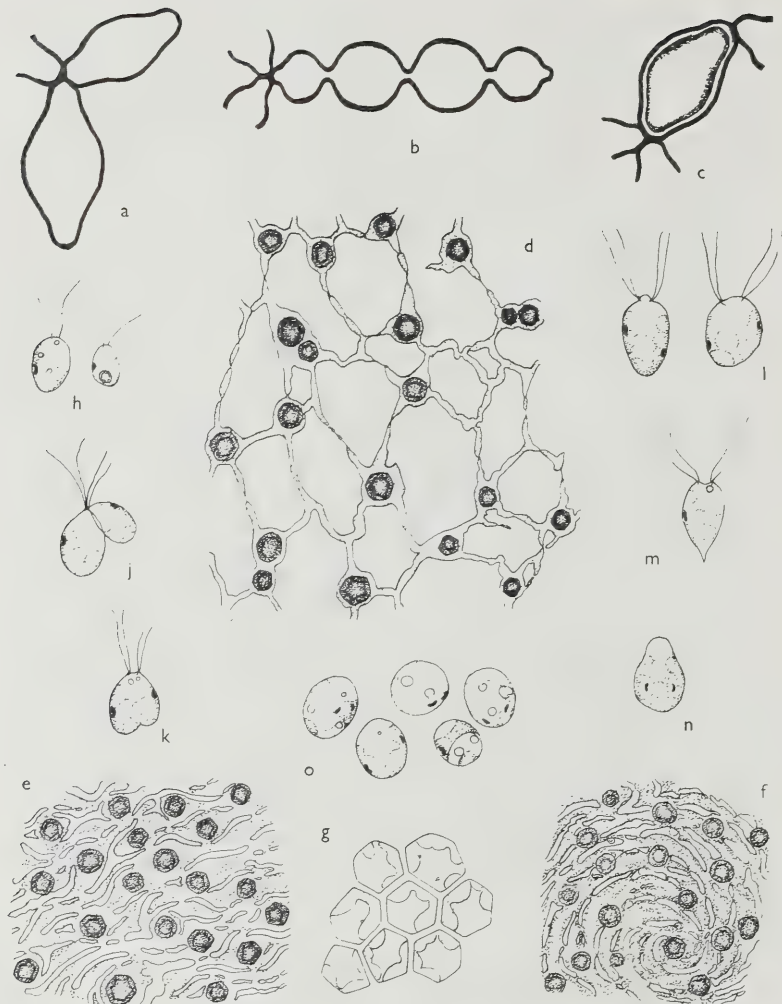


FIG. 7.—*H. africanum*. Young coenocytes. Reproduction. a—c, young developing coenocytes: a, fusiform; b, beaded; and c, already forming swarmer; d, chloroplast of adult, starved net; e, f, preparation for division; e, fenestrations coalescing; and f, "whorls" in chloroplast prior to break up into separate plastids; g, cleavage complete, protoplasts of zooids organized; h, gametes of two sizes; j—k, conjugation; l—n, zygotes; l and m, still active showing changing shapes; n, just coming to rest, flagella withdrawn, rounding off not complete; o, zygotes at rest, eyespots still present.

a—c $\times 125$; d—f $\times 500$; g, h $\times 1000$; j—o $\times 1200$.

apprehension and that, on the contrary, quite small coenocytes might produce swarms; the smallest recorded as swarming measured 538μ long by 129μ wide in the centre of the coenocyte (Fig. 7, c). Further, in general, in culture it is easier to induce small coenocytes to reproduce than the larger ones. Hence throughout this work small coenocytes have generally been used, particularly in agar-plate cultures where they are much the most convenient to work with. Fragments of small nets with still fusiform coenocytes, the largest under 1 mm. in length, isolated coenocytes of about the same size but usually spherical, and occasionally "beaded" coenocytes, such as those referred to above where each bead was approximately 80μ in diameter when swarming took place, were generally used on the agar plates; although coenocytes of as much as 4 mm. in diameter have been induced to swarm on agar, such large coenocytes are unsuitable for this method of culture. Not only is microscopical examination difficult without crushing the sphere but, in addition, there is always the danger of collapse since so large a part of the surface is exposed.

In the case of liquid cultures naturally this does not apply and coenocytes of any size may be induced to swarm, sometimes merely by bringing them into the laboratory but more often changing the medium, sometimes several times, is necessary.

(b) *Changes Leading to Division.*

The changes in the protoplast preliminary to division follow the same sequence as in the two other species investigated—the increase in the number and size of the fenestrations, their arrangement into rows (Fig. 7, e), the development of "whorls" in the chloroplast (Fig. 7, f) and the final breaking up of the pigmented layer into small rounded granular chloroplasts accompanied by the corrosion and ultimate disappearance of the pyrenoids—all follow the same pattern without any marked differences, except for the somewhat more extensive fenestration or vacuolation characteristic of the vegetative condition of the chloroplast and a slightly coarser grain in the whorled stage.

The pavement stage is fine and rather less marked than in the case of daughter-net formation in *H. reticulatum*. Measurements made of polygonal areas vary; in some cases a diameter of only 4μ was recorded; more frequently measurements (always with the 1/12 inch oil-immersion lens) gave larger dimensions, $6-6.5\mu$ (Fig. 7, g). A much larger number of measurements of numerous dividing coenocytes, however, must be made before it is possible to state with certainty that the difference is a definite one, characteristic of different coenocytes and, further, whether this difference can be correlated, as seems probable, with the marked

anisogamy described below. Division may take place in coenocytes which have turned to pale gold in colour as well as in the normal green coenocytes.

As the process nears completion the coenocyte becomes opaque and densely granular in appearance: this is accentuated as swarming begins until the whole surface assumes a curious greyish tinge. Brought under the low power, enormous numbers of swimmers may be seen moving actively in the peripheral zone of the coenocyte and although the vacuolar membrane has not been observed, all the appearances point to the conclusion that it must remain intact and operative, at any rate during the earlier stages of swarming.

(c) *The Motile Stage—Liberation.*

Normally swarming takes place simultaneously throughout the whole protoplast but occasionally cases have been observed where part of the protoplast of a coenocyte was still undivided while the rest had formed swimmers which were in active movement. Although such cases were probably pathological, they are yet of interest.

Mode of liberation was not determined, no definite pore was ever seen. As liberation proceeds, the coenocyte, which at first retains its turgidity, tends to collapse: this perhaps indicates collapse or disappearance of the central vacuole and is particularly noticeable in large isolated coenocytes. In water, the empty wall persists for some time and may retain or recover its spherical form after the swimmers have escaped and very often serves as a convenient surface on which the hyphospores are formed. In such cases its remains, part often covered with a sheet of hyphospores, will eventually fall to the bottom of the pool and get buried in the upper layers of the soil on its floor.

2. The Swimmers.

(a) *Size and Conjugation; Anisogamy.*

The swimmers are similar in structure to those of *H. reticulatum* and *H. patenaeforme* but show marked anisogamy; even at comparatively low powers of magnification it is obvious that some of the swimmers are considerably larger than others, the smaller $3 \times 4\mu$ to $4 \times 5\mu$, the larger $4.5 \times 7\mu$ to $5 \times 7.5\mu$. flagella a little more than body length in each case (Fig. 7, h). The eyespot is elongated and usually conspicuous.

Where conjugation was watched it was always between a large and a small swimmer (Fig. 7, j). It was not unusual to see gametes of the same size come together, play round one another for a time and then separate: in no case observed did conjugation take place between two gametes of the same size.

There is a certain amount of metabolic change in shape; often the anterior end elongates slightly into a small beak and the posterior pole may form a small apiculus, both often persisting for a short time only, after which the projection concerned was withdrawn and the pole rounded off once more. Such changes were often more marked in the planozygote than in the gametes (Fig. 7, l, m).

(b) *The Planozygote.*

The planozygote is a little larger than the larger gamete forming it ($5.5 \times 7.6\mu$ with flagella $7.4-9\mu$) and remains active for a short while. Union usually starts at the anterior apex and proceeds backward until complete but usually the points of insertion of the two pairs of flagella remain distinct (Fig. 7, j—m).

In some cases swarming continued for many hours, often until the day following liberation; usually when this happened agar-plate inoculations were a failure and few, if any, spores developed, whether owing to absence of conjugation or for some other undetermined cause is unknown, but, in general, examination of samples from the swarmers concerned failed to reveal the presence of zygotes and this suggests the possibility that only one type of gamete was present. Contrary to expectation, no authentic cases of azygote formation, that is of the formation of hypnospores without conjugation, were observed in the earlier cultures.

Swarmers from golden coenocytes usually showed a curiously lumpy structure in the posterior part, with several oil droplets in the anterior half and pale yellowish chloroplasts. The resultant hypnospores were also pale yellowish or golden in colour when first formed. On agar plates or after addition of fresh culture solution the colour soon changed to green.

3. The Hypnospores.

(a) *Formation and Development.*

Whatever the source of the swarmers, whether from nets collected in the field or raised in culture, once hypnospores had been formed their subsequent behaviour followed similar lines. As a general rule, if hypnospores were formed at all, they were formed soon after liberation of the swarmers. Conjugation takes place rapidly and the planozygotes soon settle down, in the process sometimes showing marked changes in shape until the final rounded form is assumed (Fig. 7, l—o). In liquid cultures spores were often found in great numbers on the outside of the walls of still undivided coenocytes as well as on those recently emptied by swarming; when formed on the sides of the glass culture vessel, it was usually just below the surface of the water.

Newly formed spores are minute ($5-6\mu$) and under high power two small eyespots can usually be seen in each (Fig. 7, 0). The chloroplasts soon merge into one, more or less completely lining the wall, and one or two pyrenoids appear. If two appear simultaneously at an early stage of development they are usually more or less equal in size; if one alone appears to begin with, those formed later are usually smaller. In this species, unlike the two species already dealt with, it is usual for developing hypnospores to form numerous pyrenoids and to become extensively vacuolated. Cultures of spores were obtained from nets collected near the Klipfontein Road which swarmed on the 4th November 1939; spores examined on the 19th showed many pyrenoids, much vacuolated contents including the chloroplasts, and had reached a size of $42-44\mu$ (Pl. XX, A, B). If conditions are favourable the spores continue to grow, reaching a considerably greater size than is usual in the other two species. Spores as large as $93-104\mu$ have been obtained while diameters varying from $62-84\mu$ are common. Most of the spores are spherical but often they become oval in outline, sometimes markedly elongate. The wall thickens considerably, varying from $3-8\mu$ in thickness and appearing hyaline while the protoplast becomes very dense, granular and deep green in colour. If the agar begins to dry, the colour changes to deep golden brown and growth is arrested, to be resumed again if fresh water or culture solution is added.

(b) Germination.

A characteristic feature which was very evident in the earlier stages of this investigation (1939-47) was the readiness with which spores of *H. africanum* germinated on the agar plates. In *H. patenaeforme* only once or twice was germination of hypnospores obtained actually on agar and never in *H. reticulatum*, whereas in all the earlier culture of *H. africanum* it was usual for a large proportion of the spores to germinate without being removed from the agar and in all such cases germination was direct, that is, germ nets were formed without the formation of zoospores and polyhedra (Pl. XX, F, G).

In 1957, however, although similar methods of culture were used, the hypnospores behaved differently. Parts of nets and separate coenocytes, collected in the ditch adjacent to Isoetes Vlei in July, were put on agar and liberated swimmers. Agar plates were inoculated with the swimmers and many hypnospores were formed and developed normally, becoming dark green, but remained smaller and less vacuolated than in the earlier experiments; none of them germinated on the agar, not even if water were added. Whether the difference in behaviour was the result of inherent

difference in the spores, or due to a different make of agar, or perhaps to extensive contamination, mainly algal, is unknown. The spores appeared perfectly healthy but only after transference to a liquid culture medium were any germinations obtained. In these again only direct germination was obtained.

All the germinations observed were direct; zoospores were never seen and polyhedra only very rarely. It was thought that these stages might possibly be present in the process of germination of younger, smaller spores and therefore attempts were made to induce spores of all ages and sizes to germinate. Similar attempts had proved eminently satisfactory in the case of both *H. patnaeforme* and *H. reticulatum* but here they failed completely. Nearly always the spores, if still small, simply continued to enlarge and eventually formed nets in exactly the same way as the larger spores, germinating directly.

In a very few cases, polyhedra were found in the cultures. Where they did occur it was usually in cultures of young spores, but what conditions are necessary for their formation, whether polyhedron-formation can be induced and whether they are preceded by the formation of zoospores, are all problems as yet unsolved. In the few cases where polyhedra were found and isolated for further development, their growth, germination and the germ nets formed by them all conformed to the usual pattern. Polyhedra have been collected in nature (Pocock, 1937, p. 275) and cultured to produce nets, the coenocytes of which ultimately developed into the round spheres characteristic of *H. africanum*, and the same is true of the several polyhedra obtained in culture (under 20 in the whole series of experiments carried on in the period 1939 to 1947) but the number of polyhedra is so small in proportion to the number of direct germinations that the latter method must be regarded as the one normal for the species and will therefore be described in more detail.

If the spores are already large, transference from agar to culture solution usually induces germination; if still small a period of enlargement follows before germination begins. The variation in the thickness of the wall is considerable, but the outer layer always hardens to form a thin but resistant surface membrane. The first visible sign of germination is the cracking of this membrane; the deep green granular protoplast enclosed in the inner wall, which is colourless, hyaline and has become more or less swollen, slips out of the ruptured outer membrane, either entirely, leaving the empty exospore behind, or partially, remaining half inside the widely opened ends of the cracked shell (Fig. 8, a—c).

If the protoplast is ready to divide germination continues immediately; if, however, the hypospore "hatches" before it is ready to germinate, a further period may elapse before germination is complete. This is

marked by the sloughing off of the outer membrane protecting the inner mucilaginous wall, which eventually forms the vesicle.

once, the final stage in germination, the formation of the germ net, being marked by the sloughing off of the outer membrane protecting the inner mucilaginous wall, which eventually forms the vesicle.

Where germination is uninterrupted, the enlargement which accompanied the initial hatching continues and the whole of the inner wall swells, enlarging to form the vesicle inside which the net is formed. If however some time has elapsed after the hatching of the spore, the new thin surface layer is thrown off and can be seen lying to one side of the vesicle wall (Fig. 8, h).

(c) *Net Formation.*

The actual mechanism of net formation is like that already described for *H. patenaeforme*, changes in the protoplast leading to the formation of the zooids following exactly the same pattern—extension of fenestration, appearance of “whorls”, division of the chloroplast and disappearance of the pyrenoids, rearrangement of the resultant plastids round the numerous nuclei and, finally, cleavage to form the pavement stage—all follow the same sequence. Here again, the resultant net in the great majority of cases is a flat plate or saucer completely one-layered (Pl. XX, C), though occasionally nets in which the central region is partially two or more layered (Pl. XX, D) or more rarely hollow nets, are seen. As the mass of zooids flattens out to form a single layer the diameter of the whole increases greatly in the plane of the net and the enlargement of the vesicle keeps pace with that of the plate of zooids. Enlargement continues at a fairly rapid rate until all movement among the zooids has ceased, after which it continues slowly as the net itself grows, until finally the wall disintegrates and the net lies free in the water. In several cases where net formation was being watched, measurements were made of net and vesicle; the following case is typical, except that often net-formation proceeded at a much more rapid rate than here:

<i>Time.</i>	<i>Vesicle.</i>	<i>Diameter of Net.</i>
1.30 a.m.	217 x 221 μ	213 μ
1.35 ,,	221 x 234 μ	225 μ
1.40 ,,	229 x 243 μ	229 μ
1.45 ,,	243 x 251 μ	243 μ
1.50 ,,	254 x 266 μ	258 μ
1.55 ,,	266 x 274 μ	266 μ

Movement of the zooids ceased entirely.

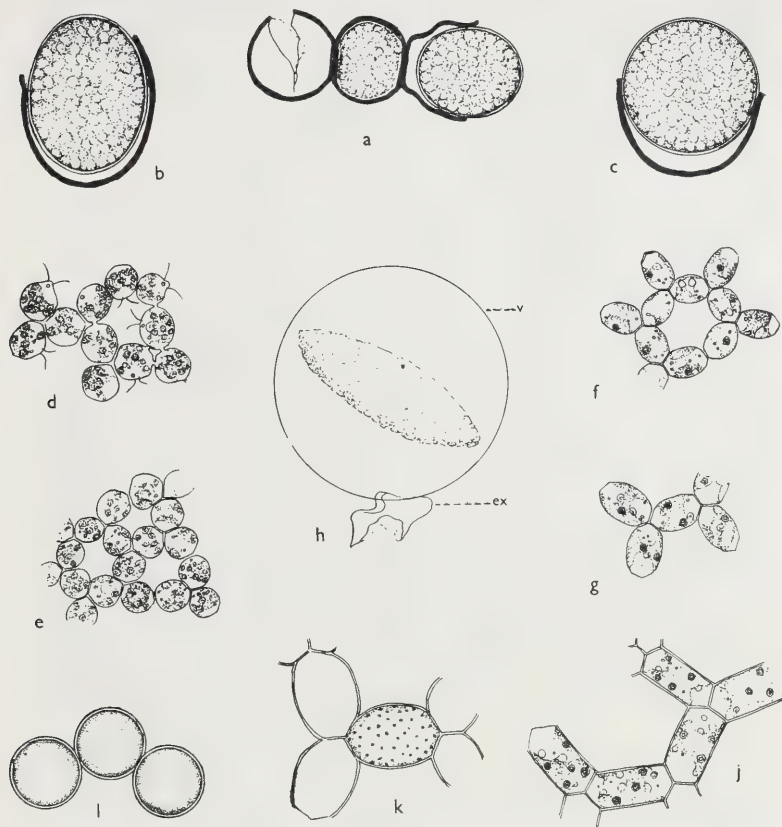


FIG. 8.—*H. africanum*. Development of hypnospor and formation of germ net. a—e, hypnospor “hatching”; d—h, formation of the germ net: d, zooids still flagellate, making contact with one another; e, net formed, chloroplasts becoming reorganized; f, cells barrel-shaped, chloroplast an incomplete equatorial girdle; g, binucleate stage, chloroplast extending; h, newly formed net, completely flat, still within the vesicle, exposure (ex) still adhering to vesicle membrane (v); i—l, developing coenocytes: j, cylindrical, chloroplast not yet extended into ends; k, beginning to swell up, chloroplast with very many pyrenoids lining the whole wall, and l, spherical, about to separate.

a—c, k $\times$ 250; d—g, j $\times$ 500; h $\times$ 125; l $\times$ 2.5.

The larger diameter of the vesicle in each case is that of the plane in which the net lies; this instance serves to illustrate a further point, that is the tendency of the vesicle to be slightly flattened along the axis

perpendicular to the plane of the net. Vesicles of considerably larger size may occur.

The net-forming zooids (Fig. 8, d) are usually about $7 \times 7\mu$ with flagella slightly less than body length (5.5μ): careful examination with the immersion lens is needed to distinguish the flagella in life and also the contractile vacuoles of which there appears to be only one per zooid. As the zooids make contact the flagella are withdrawn but here again the contractile vacuoles continue to pulsate and are for a time even more conspicuous than when the zooids were motile: soon however they too disappear.

In some cultures where conditions were not good, there was considerable irregularity in the formation of the zooids, resulting in marked variation in size. In one such case, while the majority of the zooids were $6-7\mu$ in diameter, among them were many larger, measuring 9, 13 or even 18μ in diameter. A further abnormality already observed occasionally in the other species was of fairly frequent occurrence in some of the *H. africanum* cultures: this was the rupturing of the vesicle wall at the distal end, the germinating spore being still attached to the outer spore wall, before net formation was complete. If the net had begun to form, the process usually continued on the floor of the culture glass (most often a watch glass), resulting in the formation of a loose irregular net by the majority of the zooids, while some failed to make contact or united in small groups. The stage at which rupture took place largely determined the degree of net-forming power possessed by such free zooids: if it happened at a very early stage the probability of a net resulting was low and degeneration usually supervened. But if the zooids had already begun to unite the majority continued the process, while those that failed to make contact often rounded off and continued to develop independently, either as hypnospores or as separate coenocytes. In the *Africanum* cultures, unfortunately, it was not possible at the time to follow the later development of such isolated coenocytes and spores formed by zooids, but there is no reason to suppose that their behaviour would differ from that described in similar cases for the other species, allowing for the idiosyncrasies of the species in question.

The number of zooids formed by germinating spores is usually much greater than reported by Yamanouchi for his cultures—"about 60 coenobia"—but it is variable and probably to a large extent dependent on external conditions. In nature and in favourable cultural conditions the normal number usually approximates to the ninth (512) or tenth (1,024) power of two. The net shown on Pl. XX, C, consisted of 512 coenocytes while other counts made in young nets are 956 and 1,012. But that the latter number does not represent the maximum number

possible, is shown by the beautiful net in Pl. XX, J, where over 1,500 coenocytes were counted without taking into consideration those parts of the net which show in the photograph as double; moreover, although the photograph shows nearly the whole net, it had been transferred from the original culture to a larger dish and in the process had been partly broken and some of the coenocytes lost.

4. Development of the Germ Net.

(a) *Changes in Shape of the Coenocyte.*

When first formed the net is indistinguishable from that of *H. patenaeforme* or, except for the larger number of coenocytes, even from the occasional flat nets of *H. reticulatum*. Immediately after union the zooids become approximately isodiametric (Fig. 8, e) but soon begin to elongate, becoming at first barrel shaped (Fig. 8, f, g) and then cylindrical (Fig. 8, j); the same series of changes characterizing developing germ nets in the other two species occurs here as the coenocytes enlarge. The cylindrical form of the coenocyte, with a girdle-shaped chloroplast in the central region and clear ends, persists for a time (Pl. XX, C, F, G) but soon, as the chloroplast extends and pyrenoids and nuclei multiply, a change becomes apparent. The coenocytes begin to swell up; becoming first barrel shaped once more but now very much larger (Fig. 8, k; Pl. XX, H), completely lined by the bright green chloroplast containing very many pyrenoids, and ultimately nearly spherical, the area of the contact surfaces decreasing progressively in proportion to the area of the surface of the whole coenocyte, which may reach a diameter of nearly a centimetre before separating from its fellows.

(b) *Dependence on the Nutritive Medium.*

In soil cultures the nets usually develop normally, the coenocytes becoming spherical while still quite small, often less than 1 mm. in diameter, and once the spherical shape is assumed a very slight disturbance serves to bring about dissociation, partial if not at first complete, so that, besides single spheres, rows and small portions of network may be found (cf. Pl. XX, J). The disturbance may be caused by a touch, as for example by some water animal, or by movement of the water, while even if left undisturbed the coenocytes will eventually separate.

Under natural conditions, therefore, the cylindrical shape of the coenocyte is a transient stage soon replaced by the spherical form. But when nets were grown from the beginning in the culture solution used successfully for the other two species, it was found that they at first developed normally but when the cylindrical coenocytes should have

begun to inflate they failed to do so or swelled up unevenly, one here and there perhaps becoming swollen while the majority remained cylindrical (Pl. XX, E). It was thought that possibly the salt content of the nutrient solution was too low for this species and addition of small quantities of dilute solutions of sodium and calcium salts were tried but without success; here and there a coenocyte might begin to inflate but the majority degenerated. Further and more critical experiments along such lines might give interesting results but for the immediate purpose of these cultural experiments a much simpler procedure was found to be effective. In preparing the soil decoction instead of using soil from De Klip or similar localities, soil from one of the areas in which *H. africanum* vleis form was used with the most gratifying results; the germ nets raised in such a culture solution made with extract from the whitish sandy soil from either the Klipfontein Road or *Isoetes* vleis developed normally, the swelling up of the coenocytes proceeding just as in natural conditions. The amount of soil extract is small (10 per cent) and it seems possible that it is some trace-element which is required rather than a different concentration of the normal salt content of the solution. Although the pH concentration of the vleis in question becomes very high as the season advances, by that time the *Hydrodictyon* has entirely disappeared. When first formed the pH concentration is very little if any above neutral.

(c) *Time Required for Net Formation.*

In a number of cultures development was followed from liberation of swarmers through development of spores, their germination to form new nets, growth and development of the latter until they themselves produced swarmers and so through two or more generations. Here, as in the other two species, it was found possible to speed up the various processes by judicious variation of conditions, particularly supply of nutriment, temperature and light. Special attention was being given to the development of the spores themselves however, and little effort was made to hasten development of the net; probably this too could be speeded up to some extent. The shortest time recorded for nets to be formed after liberation of the swarmers was 10 days, while it was quite common for net formation to be completed within two weeks of liberation. In every case, however, only a small proportion of the spores, whether in liquid culture or on agar, germinated so promptly, the period of germination often being spread over some days. This was particularly well seen in agar-plate cultures, where the spores were germinating on the agar; if the nets were removed as they were formed it was evident that net formation continued for some time, then ceased. Addition of fresh culture solution to the plate or removal of the remaining spores to liquid usually

produced a second crop of nets and this procedure could be repeated several times. As the culture ages, the spores tend to turn golden brown and become slower in germinating in response to changes in their surroundings; the colour change may be reversed in the early stages of germination or the germinating spores may to some extent retain their brown colour. Spores have been kept on agar for over seven months and then successfully germinated.

The differential rate of growth and germination of spores originating from the same net or part of a net is of obvious importance in ensuring the survival of some at least of the spores, possibly over several seasons. This importance can hardly be over-estimated where an area such as the Cape Flats is concerned. Given certain conditions, such as scanty rains followed by sudden and exceptional heat or unseasonal and persistent "south-easters", vleis which normally last for months may dry up completely in a few days, before there has been time for completion of the life cycle to produce a fresh crop of hypnosporos. It is therefore to be expected that the hypnosporos must have a viability extending over several years; further, the small size of the newly formed hypnosporos no doubt contributes to their survival, buried in the soil as the vlei dries up, and thus to their longevity.

Maturation of the net in culture is comparatively slow; the shortest time noted was 34 days but in most cases nets took from 7 to 12 weeks to mature. Here again, however, there is an interesting time-lag in development of the coenocytes; whereas some may form swarms when less than 1 mm. in diameter others may not reproduce until 4—5 mm. or more, even 10—15 mm. in diameter, and further, as in the case of germination of spores, there is marked variation in behaviour of coenocytes even of the same age. In the series of cultures made late in 1939 and early in 1940, some of the coenocytes collected on the Klipfontein Road swarmed immediately, others persisted for several months before they finally swarmed. In the longest consecutive set of cultures (Series I, D) three generations were obtained in nine months (10th October 1939 to 11th July 1940).

In nature, under favourable conditions, such as a sequence of warm sunny days immediately following the first filling of the vleis by the winter rains, enlargement and germination of the spores and development of the nets to maturity must often be completed in a few weeks. The size of the nets developed in the field is considerably larger than that of any obtained in culture, though such large nets as those originally found in Valkenberg Vlei are rarely seen. Usually the nets break up long before reaching any comparable size, the coenocytes continuing to develop separately growing into the well-known green "marbles". In the ditch

along the side of Isoetes Vlei the free coenocytes may collect to a depth of several inches above the floor of the ditch, while below the larger coenocytes are smaller younger ones, usually a deeper green, and beneath them again may still be young nets or parts of nets.

Whether there is more than one crop of nets in a season seems doubtful; usually the development of the initial crop is spread over some weeks and there is never the enormous mass of nets which may be produced by *H. patenaeforme*, for instance at Groen Vlei, but there is no inherent reason why there should not be more than one crop of *H. africanum* per season. Probably the limiting factor is the increasing pH concentration in the *africanus* vleis as the season advances, inhibiting germination of the spores and consequently a fresh crop of nets, unless as may very occasionally happen, late rains should refill the vleis, incidentally lowering the pH concentration in so doing.

It seems probable that the normal perennating state consists of large aggregates of minute hypnospores (often as small as 6μ) collected on empty coenocyte walls, fragments of plant detritus and particularly on soil particles where the hygroscopic water of the soil would preserve them from dessication, all finally buried in the fine silt characteristic of this type of vlei. As the vlei dries up the covering of small angiosperms present, even when the vlei is full, grows thicker and taller adding a living protection to the spores lying buried in the soil. When the first rains come, it is possible that the early stages of enlargement of the spores may begin in the damp soil, to be completed when the vlei actually forms. There is much scope for critical field experiments as well as for further cultural work on this interesting and comparatively little known species.

5. Examples of Cultures of *Hydrodictyon Africanum*.

(a) Series I. September 1939 to July 1940.

Nets of *H. africanum* were collected from vleis on the Klipfontein Road in the first week in September. Some swarmed soon after being brought into the laboratory. Others were washed and put into culture solution and later portions of nets or isolated coenocytes were used as wanted. The following are examples of a few of the many cultures made from this material:

A.

- | | |
|-------------|---|
| | Coenocytes washed and isolated in a tube of culture solution. |
| 9 Oct. a.m. | Washed, transferred to fresh solution. |
| 10 " | Swarmers liberated. Drops placed on agar. |
| 17 " | Many spores large, $44 \times 46\mu$, — 49μ , others still small, 22μ , although not crowded. Larger spores vacuolated with 1 pyrenoid. Some transferred to culture solution; further observations are of these: |
| 19 " | Spores becoming congested dark green with numerous pyrenoids. Distilled water added. |

- 20 " No sign of polyhedra, but many newly formed nets, rather irregular but all one layered.
 One case of net formation within the spore, i.e. without formation of a vesicle, noted.

Time from liberation of swarms to formation of net: 10 days.

B.

- 10 Oct. Swarms to agar. 4.30 p.m.
 3 Nov. Many nets from larger spores (24 days).
 Remainder turning golden.
 6 " Golden spores (diameter— 62μ) to culture solution in watch glass.
 7 " Majority hatched; a few nets, thin outer coat thrown off in net formation.
 Two polyhedra, 1 normal, 1 rounded with short blunt spines.

C.

- Coenocytes from same source isolated in tube of culture solution.
 29 Jan. 1940 Swarms liberated; put on agar, 3.30 p.m.
 30 " Still motile, 2 a.m.
 21 Feb. Spores small, green. Transferred to culture solution.
 1 March Culture dry; 25 per cent culture solution added. Spores with 2 to many pyrenoids.
 5 " Spores green.
 7 " Distilled water added.
 13 " Fifty per cent of culture solution added.
 1 April Some nets.

This culture is illustrative of the several changes of medium which were sometimes necessary to induce germination, particularly where the spores were either old or had been more or less dried.

D.

- 1 Sept. 1939 Nets collected Klipfontein Road put in culture solution.
 10 Oct. Swarms liberated; put on agar.
 23 " Germ nets on agar. (1) First generation, 13 days.
 27 " Germ nets transferred to culture solution.
 19 Jan. 1940 Germ net, 1.5 cm. in diameter put on agar.
 22 " Some coenocytes ($538 \times 129\mu$, etc.) swarming, many golden green, no pyrenoids visible, others dense green still with pyrenoids.
 Swarms put on agar; "clumping" marked, conjugation seen. 9.30 p.m.
 2 March Spores put in culture solution.
 17 April Nets formed. (2) Second generation.
 Nets transferred to culture solution.
 7 June Nets 52 days old, some coenocytes swarming.
 Swarms to agar.
 28 " Spores to culture solution. Mostly large with many pyrenoids.
 11 July Some nets formed direct on agar.
 (3) Third generation.

Here 3 generations of nets were obtained in the course of 9 months.

E. (Sub-culture from D.)

- 10 Oct. 1939 Swarms to agar, 3 p.m.
 23 " Some nets, on agar. (1) 13 days.
 Nets removed.
 2 Nov. Many nets on agar. Nets removed. Washed with distilled water.
 17 " Culture solution added.
 25 Dec. Culture solution with Klipfontein Road soil extract added.
 11 Jan. 1940 Culture solution added.
 2 March Spores to watch glass of culture solution.

- 10 " Many nets.
 16 " Culture solution added.
 10 April Culture dry; spores golden. Culture solution added.
 17 " A few nets, some spores hatching, many dead.
 3 June Many nets, all sizes, larger ones very pale.

In this culture which suffered many vicissitudes, spores were induced to germinate on the agar plate over a period of over 7 months.

(b) *Series II*

Nets raised at Grahamstown in students' cultures of soil from *Isoetes Vlei* on the Cape Flats. Nets appeared in the cultures in August. Some were removed and transferred to culture solution made with extract from *Isoetes Vlei* soil. Those left in the original cultures eventually showed starvation phenomena, probably due to too intense insolation, where the cultures had been exposed to sunlight; in others kept in the shade they developed well, remaining bright green. Coenocytes some 2 mm. or more in diameter began to dissociate.

A.

- Sept. 1947 Coenocytes washed and put on agar plate.
 5 Oct. Swarming. Swarmers pipetted off and put in watch glass of culture solution, then drops put on agar. Conjugation with marked anisogamy general in both culture solution and on agar.
 6 " Hypnosporos rounding off.
 7 " Hypnosporos rather sparse, scattered.
 10 " Enlarging well; a good culture, little contamination except for diatoms; all inoculations show spores scattered over whole area of drop with some congestion round edge, indicating absence of marked phototaxis.
 18 " Some nets formed on agar, direct from spores. (13 days.)
 15 Nov. Spores loosened from agar, put in culture solution.
 Starved, nearly colourless nets formed on agar also removed to culture solution.
 6 Dec. Some of these nets completely recovered, bright green, others dead. Coenocytes still cylindrical. Many newly formed nets.

B.

- ? 18 Sept. 1947 Net from soil culture put on agar.
 25 " Some coenocytes swarming. Swarmers put on agar in drops of culture solution.
 11 Oct. Very good development.
 16 Nov. Some spores large, golden, others small, still green. Some removed from agar and put in culture solution.
 18 " Some nets.
 Three polyhedra seen.

The polyhedra were isolated in culture solution but owing to difficulties in cultural work at the time, it was not possible to carry on their development.

C. "Starved" net culture.

- 17 Sept. 1947 Small "starved" net from soil culture transferred to fresh culture solution in shaded window. When isolated, chloroplast broken up into attenuated strands forming incomplete net-work with very large meshes, some strands themselves breaking up into smaller granules. Net very pale, yellowish.
 27 " Colour strengthening, chloroplast net-work less attenuated (Fig. 48), size increasing.
 30 " Part transferred to agar.
 8 Oct. In most coenocytes chloroplast continuous with fine fenestrations, in others still vacuolated.
 28 " Many small hypnosporos already formed, some on agar some on coenocyte walls. Other coenocytes uniformly granular.

- 30 " Some coenocytes swarming, some in pavement stage (polygons 6μ diam.), others still granular, still others with pyrenoids; some had degenerated. Hypnospores $6-12\mu$ in lines along the coenocytes and on the agar.

N.B. In some of the coenocytes part of the protoplast had formed swarmers while a part was undivided and showed signs of degeneration. The swarmers were very active and apparently quite normal.

D.

- 20 Sept. 1947 Young net put on agar.
26 " a.m. Swarmers liberated. As the swarmers escaped clumping was very marked; later conjugating pairs were noticeable.
 p.m. Coenocytes in the granular stage in the morning reached the pavement stage in early afternoon and swarmed about 4.30 p.m. Contraction of vacuolar membrane marked, swarmers active in peripheral layer separated from outer wall by a wide hyaline zone (cf. Fig. 7, c).

(c) Series III.

- July 1957 Portions of young nets and single coenocytes collected at Isoetes Vlei, in ditch along roadside.
Aug.-Sept. Some nets were kept in culture solution, others put on agar. After some days in many of the smaller coenocytes swarmer formation was completed and liberation followed. Swarmers transferred to fresh agar plates settled down and formed hypnospores.
 The hypnospores enlarged slowly, majority deep green, granular in appearance.

N.B. No hypnospores reached the large size obtained in the earlier cultures and none germinated on the agar. Transferred to culture solution some germinated directly to form germ nets. The cultures were repeated several times with the same results.

Contamination of the plates was bad, chiefly algal, a few fungal, especially a small species of *Aspergillus*, the hyphae spreading over and among the hypnospores which were not attacked by the fungus.

DISCUSSION.

1. REPRODUCTION IN *Hydrodictyon patenaeforme* AND *H. africanum*.

The absence of asexual reproduction by means of daughter-net formation in both the South African species of *Hydrodictyon* is so diametrically opposed to the normal course of development in *H. reticulatum*, where this form of non-sexual reproduction dominates the life cycle, that it seemed logical to expect that in these species, too, some form of asexual reproduction would be found. Extensive observations in the field through many years, as well as numerous cultures carried on in the laboratory, failed to reveal any trace of daughter-net formation in either species and a similar state of affairs has been reported from other parts of the world, for example by Tutin from Lake Titicaca. Everything confirms the original impression that daughter-net formation does not occur in these species and that they must depend for their propagation entirely on motile cells liberated into the water.

The experiments here described have shown that in *H. patenaeforme*, while the swarmers can and do behave as gametes, conjugating readily with one another, they may also behave as accessory spores, settling down without conjugation to form a resting spore essentially similar in all but genetical constitution to that formed by the planozygote.

The question as to the nature of these swarmers then arises. It is conceivable that here non-sexual zooids are formed which, instead of uniting with one another within the parent coenocyte to form a daughter net, are liberated into the water and there form neutral haploid hypnospores and that such zooids are essentially different from gametes. Considerable variation has been noticed in the swarmers in size, structure and response to light. At first it was thought that two distinct forms of swarmers were produced—small gametes with an oblique undivided chloroplast enclosing a rudimentary pyrenoid and with a distinct eyespot, and slightly larger asexual zooids with several chloroplasts, no pyrenoid and usually but not always an eyespot. More extensive observations however tend to show that there is no definite line of demarcation between the two types of swarmers: the form of the chloroplast in particular is a very variable character depending largely on external conditions. Owing to their rapidity of movement and small size it has not so far proved possible to reach a satisfactory conclusion as to the presence or absence of two distinct types of swarmers.

Normally in a healthy coenocyte the size of the swarmers is remarkably uniform: if however conditions are unfavourable either during the maturation of the coenocyte or during swarmer formation—as for instance overcrowding, contamination of the culture—division may not proceed so regularly, resulting in considerable variation in size. Or too intense insolation may cause changes in the chloroplast. In the former case the larger swarmers which may result, even though uninucleate, are equivalent to two or more of normal size, arising through the failure of some of the nuclei to divide simultaneously with their fellows. Such gigantic swarmers are however rare.

Perhaps the best view to take is to regard all swarmers in this species as facultative gametes of so nicely balanced a nature that slight variations in either environmental or developmental conditions may determine whether they are to behave as gametes or as accessory spores. In the laboratory culture conditions appeared on the whole to be more favourable to the latter mode of behaviour and in many of the cultures in which germination of the resting spores was investigated, azygospores predominated over zygozspores, although in nearly all some zygozspores were found among the germinating hypnospores. In nature, the relation between the two types of spore formation is still undetermined; conjuga-

tion has often been seen in material brought in from the field, yet in plate cultures made of swarmers collected, for example, in Groen Vlei, zygotes predominated.

Whatever view is taken, it is certain that the second type of reproduction is one which is normal to the species, replacing daughter-net formation, and occurring regularly in the field. The appropriate nomenclature presented a difficulty; the terms azygospore and azygotie are used here for convenience in distinguishing those hypnospores which have been formed without conjugation but as "azygospore" is sometimes used as equivalent to parthenospore, abnormality is implicit in its application and therefore its use is not ideal as applied to a reproductive cell which here would appear to be the normal, natural form of asexual reproduction.

In the case of *H. africanum* it was expected that the same type of asexual reproduction would be found, particularly in the light of the state of affairs reported by Miss Wigglesworth (1927-28) who found that spores of this species formed in culture in Manchester germinated direct. The present investigation amply confirms her results, the formation of polyhedra, although recorded, being extremely rare. But so far as could be determined, the hypnospores appear to result usually from conjugation. In the earlier experiments all attempt to show that in this species too swarmers may settle down without conjugation to form spores were unsuccessful. In the 1957 experiments, however, although resting spores were formed, conjugation was not seen, and it is possible that in these cultures a similar form of asexual reproduction to that observed in *H. patnaeforme* occurred. Further investigation is needed to confirm or refute this possibility.

2. SWARMERS OF *Hydrodictyon reticulatum*.

In *Hydrodictyon reticulatum*, on the other hand, it became clear very early in these experiments that liberated swarmers were definitely of two kinds. In addition to the small swarmers or gametes ($4-5\mu \times 5-7\mu$)—the "microgonidia" of Braun (1851, p. 21; 1855, p. 63) and other workers—and the net forming zooids—"macrogonidia" of Braun (l.c.)—which unite to form the daughter net within the parent coenocyte, this species produces yet another type of motile cell. These are identical in origin and form with the zooids, but instead of uniting to form a daughter net, they are liberated into the water and there behave as swarmers, sometimes conjugating, sometimes forming hypnospores direct without previous conjugation, i.e. they may behave either as gametes or as accessory spores. They are slightly larger than true gametes, have more numerous and more distinctly separated chloroplasts and usually no eyespot (Fig. 6, p.). That these larger swarmers and the net-forming zooids

are, in fact, identical, differing only in behaviour, was nicely demonstrated in a coenocyte the wall of which had been ruptured near one end while net formation was actually in progress. In the uninjured part of the coenocyte a large proportion of the zooids had formed a normal net, closed at the intact end, open and ragged near the torn part, from which a number of zooids had escaped. Of these some settled down and formed hypnospores while others, although separated from their net-forming companions, had yet progressed so far in the net-forming condition that they continued development into elongated coenocytes although each developed separately (Pl. XIX, C). Other similar cases have since been observed.

As a general rule, when a coenocyte becomes reproductive, all the biflagellate bodies formed in it either unite to form a net (though occasionally one here and there fails to unite and develops separately) or all escape into the water. Klebs (1896, p. 158) states that it is impossible for one and the same coenocyte to produce both types of reproductive cells. Hence the case described here is particularly interesting since it shows that, contrary to Klebs's statement, such behaviour, although unusual, is not impossible. Being on agar the subsequent development could be watched—net, coenocytes and hypnospores all developed normally. But Klebs had recognized only one type of liberated swimmers, the smaller gametes, and his statement appears to be true so far as such true gametes are concerned. They are about half the size of zooids and so far have never been observed uniting to form a net—the small swimmers are always liberated or, if they fail to escape as sometimes happens, they do not unite but develop into rounded resting spores within the coenocyte. Conjugation may take place within the parent cell.

Many cases were seen where, instead of ending in net formation, the movement of the zooids strengthened and the zooids collected along the centre of the coenocyte but failed to escape, rounding off and forming a compact mass of spores inside the coenocyte. Such spore masses have been matured and germinated and in every case proved to consist of both zygotes and azygotes.

Although copulation was seldom observed among these liberated zooids, conjugating individuals were occasionally seen and in iodine-fixed material a few quadriflagellate planozygotes were usually found. Further, on germination, zygotes were always present no matter how the spores had arisen. Hence it would appear that, while such larger swimmers may develop directly into hypnospores, they may also behave like gametes and conjugate before rounding off, unless the apparently diploid constitution of the resultant spores is attributable to some other cause than normal conjugation. Klebs (l.c., p. 168) states that whereas the swimmers

of *H. reticulatum* liberated into the water were obviously gametes which conjugated in pairs to form zygotes, he could under certain conditions, for example by putting single coenocytes which were already well advanced in gamete formation into a drop of 1 per cent culture solution in a moist chamber, bring about inhibition of conjugation, whereupon the gametes settled down and without copulation formed resting spores essentially similar in appearance to young zygospore. He was however unable to induce these parthenospores to develop further. He concludes that while it was obviously possible for individual gametes to form walled spores without copulation, the conditions in which these spores could develop further remained unknown. Earlier in the same paper (p. 158) he emphasizes the essential similarity of the "zoospores" (that is, of the units participating in net formation, here called "zooids", not those formed on germination of the spore) and gametes. There is nothing to show whether the swimmers Klebs was here dealing with were true gametes or zooids, but in the present series of experiments it has been found that hypnospores of non-sexual origin are of common occurrence, that they develop just as do zygospores except that on germination no division takes place, each resting spore producing a single zoospore. In all germination experiments both azygotes and zygotes were present, but whereas in *H. patenaeforme* the former predominated, in *H. reticulatum* the reverse was the case, zygospores always being the more numerous.

What determines the degree to which division in the coenocyte proceeds remains to be discovered. Vigorous growth brought about by ample food supply and space most often resulted in daughter-net formation, succeeded by liberation of swimmers as the food supply diminished. On the other hand, gamete formation was most often observed in old cultures—i.e. those in which conditions had been such as to retard development; on a return to more favourable conditions net formation sometimes supervened, sometimes gamete formation. Further, liberation of zooids appeared to be of common occurrence in winter and spring when the development of hypnospores was most rapid, while gamete formation was common later in the season in summer; whether this relation is actually seasonal needs further investigation. It seems probable that the difference between night and day temperature plays an important part, perhaps more so than the actual higher summer temperature.

Whatever may be the determining factor which decides to what degree division of the protoplast shall proceed, the result is that in the one case the stage at which it stops produces zooids, in the other it proceeds a degree further and gametes are produced. The zooids are further differentiated by their behaviour, either re-uniting to form a net or becoming increasingly active and in general escaping from the parent coenocyte to

swarm in the water much as do the gametes. The zooids therefore may be regarded as essentially accessory spores which may either unite to form a net, round off to form asexual resting spores or may even, on occasion, function as gametes. The gametes, on the other hand, normally conjugate to form zygospores; should conjugation fail, however, they may form hypnospores without conjugation (azygotic), but this is comparatively rare and, unlike the larger zooids, they never unite to form a daughter net.

3. NATURE OF THE HYPNOSPORE.

Whatever the mode of formation, the subsequent history of the hypnospore follows along similar lines except that in some, presumably haploid, no nuclear division precedes zoospore formation, whereas in others, presumably diploid, there are typically two successive divisions, normally indicative of reduction division, resulting in four zoospores. In addition, at times other numbers of zoospores may be formed from a single hypnospore.

In all the experiments so far made, it has been found that in *H. patenaeforme* hypnospores which on germination form a single zoospore predominate over those which divide, while in *H. reticulatum* the reverse is the case, even when the spores have been formed by zooids settling down within the coenocyte. Before this occurs the zooids usually swarm actively in the centre of the coenocyte so that the possibility of copulation is not excluded—among gametes it certainly may take place within the parent wall; active copulation has repeatedly been seen inside partly emptied coenocytes. Sometimes, however, the mass of separated zooids contracts and the zooids round off to form hypnospores without ever being actively motile. In such cases it is difficult to see how conjugation could take place and probably the resultant spores are mostly if not all azygotic.

Throughout this paper the assumption is made that division of the spore protoplast in germination into four parts indicates reduction division, that the spores showing it are diploid and that they are zygotes resulting from the conjugation of either true gametes or zooids functioning as gametes. This provided a convenient working hypothesis, but it must be emphasized that a thorough cytological investigation of all stages in the life history of all the species, especially *H. africanum*, is still needed.

Of the cases where the number of zoospores formed is other than one or four, those cases where more than four are formed are easily explained. If the zygospores has reached a large size before germinating, eight zoospores are usually formed, the two reduction divisions being followed by a third, which adjusts the size of the resultant zoospores. If one or more

of the four parts should fail to divide, seven, six or five zoospores may result. The zoospores formed from zygotes average about the same size and the extra divisions merely compensate for the larger size of the respective zoospores.

Where fewer than four zoospores are formed the explanation in most cases is that cleavage has been incomplete or even totally absent, whether this be primary or secondary. A very common case is that where two zoospores only are formed; some cultures showed zygosporos dividing normally, the first cleavage complete, the second beginning. But after proceeding a short way, the second cleavage stopped and the protoplasts reunited, resulting in the division of the protoplast into two equal parts each of which formed a zoospore. In life it is difficult to distinguish the number of flagella, but in those cases where such zoospores were killed with iodine, two pairs of flagella and two nuclei could be seen in each and it is probable that this is always the case. If one of the parts resulting from the first cleavage fails to divide, three zoospores, one double the size of the other two and as in the last case binucleate with two pairs of flagella, result (Fig. 3, d—f), and similarly other numbers can be explained. Finally, cases occur where no cleavage follows nuclear division, or if it begins it is incipient only and soon disappears. The result is a single, quadrinucleate zoospore with four pairs of flagella. These and similar observations fully confirm and amplify those recorded by Pringsheim (1860, p. 8) who found that from two to five zoospores might be formed in *H. reticulatum*.

4. MULTIPLE AND ABNORMAL FUSIONS.

Abnormal figures may also occur among gametes. Sometimes these are obviously attributable to incomplete separation during the formation of the gametes but in other cases there may actually be fusion between more than two gametes. One such case was watched actually in the process of fusing (December 1940). Several coenocytes of *H. reticulatum* on the slide were liberating gametes and conjugation was proceeding vigorously. One actively moving group consisted of two rather large swimmers already partially fused with a third smaller one in contact at the apices; soon this one too was seen to be fusing with the first pair. Fusion was complete within a few seconds, after which the whole composite body proceeded to round off just as does a normal zygote. Details of flagella and contractile vacuoles in this case could not be made out owing to the position, the apex being somewhat depressed. It would be extremely interesting to follow the subsequent history of such resting spores. Similar multiple fusions were observed by Mainx (1931, p. 500).

Normally, fusion begins at the apices of the gametes but, in addition, various abnormalities have been noted; the case shown in Fig. 6, c, might possibly be abnormal fusion but more probably resulted from incomplete septation during the formation of the gametes, but there is some evidence that, however they may have arisen, such pairs may ultimately fuse completely. The structures shown in Fig. 6, e, were not uncommon in some cultures and may have arisen similarly from incomplete cleavage leading to an abnormal type of fusion.

5. HAPLOID AND DIPLOID PHASES.

Such abnormalities excluded, the cytological history of *Hydrodictyon* emerges clearly from the observations here described:

The net is haploid. In *H. reticulatum* this part of the haploid phase may be repeated indefinitely by asexually formed daughter nets, but in *H. patenaeforme* and *H. africanum* the latter form of reproduction is unknown; in the former it is replaced by the formation of hypnospores by swimmers without conjugation, the resultant hypnospores though azygotie being indistinguishable from zygosporcs, except in their subsequent behaviour. Similar azygosporcs may be formed in *H. reticulatum* but more rarely. In *H. africanum* they have not been fully demonstrated but probably here too they occur. The haploid phase terminates in conjugation of gametes to form the zygote, which initiates the diploid phase and, indeed, constitutes the only cell in this phase. But although the diploid phase consists of a single cell it may have a prolonged existence during which it passes through two distinct habit forms, the first short, as the motile planozygote, the second vegetative, and often prolonged. When first formed the planozygote is similar in size and form to the haploid swimmer except that its nucleus and flagellary apparatus are duplicated. How soon nuclear fusion follows cytoplasmic fusion is as yet unknown. Observation of newly formed planozygotes has shown in many cases that the flagella and contractile vacuoles of the two conjugating gametes remain separate and distinct up to the time of rounding off, that is, when the motile is replaced by the quiescent state. Probably nuclear fusion ends the motile phase, although the eyespots still persist for some time. When it has been possible to distinguish nuclei in young resting spores they have always proved to be uninucleate.

The rounding off of the zygote initiates the second stage in the history of the diploid phase which itself again shows two distinct periods, one of rest the other of growth, before entering on the final stage, that of germination. The zygote may at once enter on a period of rest after the secretion of a wall, and probably in nature this is the more usual pro-

cedure. When coming to rest the planozygotes tend to collect on any available surface whether of soil particles or debris, often in great numbers; they thus act as a mutual protection against dessication in addition to the protection afforded by the hygroscopic water of the soil and in this state they may survive prolonged periods of drought extending over many months if not years—in nature probably terminated by the advent of the next wet season. If however conditions are favourable as on agar plates, the newly formed zygotes enter a period of growth immediately; if not, the growth period is postponed until the resumption of activity after the resting period. Whether this immediately follows formation or is postponed until just prior to germination, it is characterized by a series of changes similar to those which obtain in any cell of the haploid phase in so far as concerns the cytoplasm, remaining uninucleate throughout.

The diploid phase ends with germination of the zygospore when division into a tetrad of nuclei (i.e. reduction division) is the normal procedure. The cytological investigation undertaken by Proskauer (1952) has confirmed what a study of the living organism had suggested, i.e. that the net is haploid and that reduction division takes place, as was to be expected, at the germination of the spore. Further, his work shows that there are rather numerous small chromosomes, apparently the same number (19 ± 1) in all three species, and that in *H. reticulatum* reduction division occurs at germination of the zygospore. This is probably true also of *H. patenaeforme*. Mainx (1931, p. 514) by progressive staining of germinating spores had previously obtained a few nuclear figures but although he judged from the form of the prophase that reduction division was taking place, chromosome counts were not possible and his results though strongly suggestive are not conclusive. Tilden (1935, p. 375) states that the "zygote and polyhedron together" represent the sporophyte but gives no reason for this view. Nothing in the present investigation suggests that this is the case; on the contrary, all the evidence we have indicates that the sporophyte or duploid phase ends with germination, that the zoospore then formed initiates the new haploid phase and that the polyhedron, also haploid, constitutes an interpolated vegetative stage, peculiar to the family. In *Pediastrum* polyhedra very similar to those of *Hydrodictyon* occur. The fact that zoospores, azygotes and even swimmers may grow directly into a coenocyte which then develops just as does the coenocyte of a net, thus entirely cutting out the polyhedron or the resting spore, serves to emphasize this fact. By its form, with thick wall and spiny processes and slightly mucilaginous outer surface, the polyhedron is protected during development from destruction by animal organisms and is well adapted to conditions of life in still or slightly moving water. In *H. africanum*, however, though occasionally

observed, the polyhedral stage seems to be normally cut out of the life cycle, and zoospores have never yet been observed.

The polyhedron again passes through a period of growth which culminates in the formation of the primary or germ net, the only net found in the life cycle of both *H. patenaeforme* and *H. africanum* but in *H. reticulatum* soon replaced by the better known cylindrical daughter nets.

6. RECURRING CYCLES IN THE LIFE HISTORY.

The life history of species of *Hydrodictyon* thus shows a repeated alternation of a vegetative non-motile stage with a reproductive motile stage which may occur thrice in the life cycle. Each motile stage, in turn gives rise to a vegetative phase characterized by growth accompanied by changes in wall and protoplast. These vegetative phases, each of which in turn ends in the production of unicellular motile bodies, are:—

1. The net itself, culminating in the formation of gametes, or zooids.
2. The hypnospore, whether haploid or diploid, culminating in the formation of zoospores.
3. The polyhedron, culminating in the formation of net-forming zooids.

The motile phase is typically unicellular and uninucleate, except in the abnormal cases already discussed: there are minor differences in size and details of structure, such as form and number of chloroplasts, presence or absence of a stigma, etc., but the general plan is the same throughout—clear apex containing the nucleus rather to one side, obliquely posterior chlorophyll-containing portion, two flagella inserted separately. In each case the motile stage is comparatively short. In the zoospore it is apparently always less than an hour, rarely as much as half an hour, sometimes lasting only a few seconds, but typically free in the water; the zoospore is the largest of the motile cells, usually deep green, with flagella which are exceedingly fine as compared with the protoplast, and no stigma. The net-forming zooids, on the other hand, whether in the adult coenocyte in *H. reticulatum* or in the germ net in all three species, are normally not liberated into the water, and have only a very restricted type of movement of comparatively short duration. Movement is longest and freest in the swarmers whether these be gametes or zooids liberated into the water, but more particularly in the case of the former which moreover are the smallest and normally have an eyespot. Here, movement is very active and may continue for hours, occasionally even as long as 24 hours, or may end in a few minutes; for example in some

December cultures where copulation was in exceedingly active progress, liberation, swarming, conjugation and rounding off of the planozygotes to form resting spores were often completed within two or three minutes of the commencement of escape from the coenocyte.

The motile stage ends in the resumption of the static condition; as movement ceases the flagella are not cast off but are withdrawn into the protoplast and a wall is secreted. In each case the vegetative stage undergoes changes which follow the same general pattern. The parallelism is particularly close in the case of the net-forming coenocyte and the polyhedron—at first uninucleate with one pyrenoid, repeated nuclear division and formation of pyrenoids results in a multinucleate, coenocytic structure. In both the protoplast passes through a similar series of changes; coalescence of the separate rounded chloroplasts usually present in the motile stage results in the formation of a single parietal chloroplast at first girdle shaped but soon lining the whole wall, enclosing a large central vacuole. The further changes which lead to division and the formation of motile cells all follow one another in the same sequence in either case.

In the hypnospor the parallelism is in some respects less close since here the cell remains uninucleate until immediately prior to the resumption of the motile state and even then nuclear divisions, if any, are limited to two or at most three. Further, the initial number of pyrenoids, whether one or two, may remain unchanged throughout the period of growth until they finally disappear. In some cases however a number of pyrenoids may develop. The chloroplast itself however undergoes very similar changes to those summarized above.

In *H. reticulatum* vegetative reproduction by means of daughter-net formation may interpolate an indefinite number of vegetative phases in the life cycle before swarmer formation. In *H. patenaeforme* this form of reproduction is replaced by liberation of zooids and formation of hypnospor without conjugation (which may also occur in *H. reticulatum*), while in *H. africanum* the second motile stage, the zoospore, is usually omitted and the succeeding vegetative stage correspondingly modified, although occasionally true polyhedra and presumably zoospores do occur.

7. MODIFYING EFFECT OF EXTERNAL CONDITIONS.

The aim of these observations and experiments has not been to study the effect of artificial conditions but to note the response of the organisms to natural changes in environment—temperature, insolation, food supply. The sequence of events in the life cycle may be modified in many ways by interaction with external conditions which may normally occur in nature and are easily observed in the laboratory. One of the most notable

variations is colour-change due to the alteration of chlorophyll to haematochrome with accompanying decrease in carbohydrate content and accumulation of fatty reserve material, sometimes associated with diminution or even complete disappearance of pyrenoids. This may occur in the net itself but is more marked in the resting spores, where it may be to some extent a normal feature of development accompanying the drying up of the surroundings. But it may be induced by several causes—deficiency in nutrient salts, intense insolation—besides the actual lack of water. In the polyhedron, if formed from a green zoospore, no haematochrome is found nor is it likely that external conditions could induce its formation without injury to the polyhedron itself. But if the zoospore which gives rise to the polyhedron was formed from a brown or golden brown spore, that is, one in which haematochrome masks the green colour, it, too, usually contains haematochrome or oily reserve coloured with haematochrome and this may be carried over into the polyhedron. Such oily reserve may persist unaltered through the development of the polyhedron and when the germ net is formed, remain unused. Germ nets of *H. patenaeforme* have frequently been obtained in which remnants of the fatty reserve of the hypnospore have survived in the form of small reddish oil droplets, held in the meshes of the young net. In most cases such nets were derived from very large brown azygotic hypnospores.

In cultures of *H. reticulatum*, as for instance in a beaker in which a single net had developed and formed daughter nets, overcrowding and consequent exhaustion of the nutrient salts resulted in partial starvation and the young nets became at first yellowish green, then golden, with much vacuolated or even divided chloroplasts; addition of fresh culture solution without further change of condition brought about a colour change back to green in a couple of days. A similar effect is caused by keeping the culture vessel in too strong light, but here the effect of the light is to speed up metabolism and thus bring about exhaustion of food material so that although removal to a more diffuse light may restore the green colour to some extent, addition of culture solution will probably also be necessary for complete restoration. The effect of intense insolation is beautifully seen in the field at Groen Vlei—as the season advances the nets forming the upper layers of the mass filling the vlei turn bright reddish gold, colouring the whole expanse of the vlei, while below, shaded by them, are green nets, the colour getting progressively deeper green with increasing depth and consequent increase in shade. In this case there is also an appreciable difference in temperature between the upper and lower layers.

A simple experiment demonstrated the relation between the colour

change and degree of insolation. In December 1940 two young nets of *H. reticulatum*, of approximately equal size and comparable in development, were placed in small beakers containing equal quantities of culture solution, A placed near a sunny window, the other, B, at the back of the laboratory. Both were at first green, and to begin with A grew more rapidly than B. Then the rate of growth in A began to fall off and the colour to become yellowish, while B continued to develop steadily; the net was now larger than A, the individual coenocytes longer, while the colour was still bright green. Equal quantities of culture solution were added, A began to turn green once more and resumed growth but again soon fell back in development and haematochrome reappeared. Kept in the dark, nets of *H. reticulatum* may remain green and viable for months, resuming growth on being brought back into the light.

Lack of water naturally causes irreparable injury in the case of the comparatively thin-walled coenocyte and polyhedron, but the effect can be well seen in spores which are able to withstand a considerable degree of drying; beakers in which spore formation has been taking place may show a film of green spores on the sides just below the waterline; as the water evaporates this begins to turn golden and as the level of the water sinks a continually widening rim of reddish gold forms above the water level, while below the colour remains green. On agar plates as the agar dries the colour change can be more exactly observed—first the bright green changes to a yellowish green then to gold. In both these cases addition of distilled water alone will often bring about a reversal of the colour change. Usually, however, addition of culture solution supplying both water and food is more effective if the drying process has not proceeded too far.

8. THE STRUCTURE OF THE CHLOROPLAST.

The form of the chloroplast in *H. reticulatum* has given rise to much controversy and considerable divergence of opinion as to its nature still exists. A careful study of its form through the succeeding stages of the life history shows that it is essentially the same in all the species and, further, that the discrepancies in the various accounts of its form are probably nearly all due to either incomplete observation or more often to observation of partially starved material; at different stages in the life history it assumes different forms, and varying external conditions affect it materially.

The earliest accounts describe the cell as uniformly green, e.g. Ray (1686, p. 81) "colore viride", Lamarck (1786, p. 81) "ses filamens sont . . . d'un verd pale". Areschoug (1842, p. 128) describes the mature cell as entirely covered with a thin green mass in which lie embedded the

"sporidia" (he appears to have mistaken the pyrenoids for reproductive bodies). Braun (1855, p. 55) gives a more detailed description and recognizes that the pyrenoids are not reproductive but are starch-containing bodies: "... cytoplasma viridi, granulose, globulis amylaceis initio unido, demum numerosissimis instructum". In the *Verjungung* Braun (1851) gives a detailed description of the successive changes in the cell leading to zooid formation in the course of which he notes the disappearance of the pyrenoids and the appearance of "helle flecken" (the nuclei) surrounded by groups of chlorophyll granules, the groups appearing as polygonal tablets and later, after the net is formed, the coalescence of the granules to form a homogeneous mass and the reappearance of the pyrenoids, but describes the "broad green girdle" of the young cell as becoming more and more divided until it assumes the form of a many meshed net (p. 184), which in the mature cell becomes granular.

Artari (1890, p. 279) concludes that the apparently distinct chlorophyll grains are not separate granules but form a continuous structure, a complex net lining the inner surface of the cell. When the "macrogonidia" are forming, the chromatophore divides into as many parts as there are nuclei (Braun's "helle flecken") and each puts out a lobed structure surrounding a nucleus. He does not believe that the chloroplast breaks up into separate granules (l.c., p. 282).

Klebs (1896, p. 135) states that in the parietal layer of the protoplast there is a chromatophore in the form of a thin continuous chlorophyll sheet containing many "Amylonkerne". In his earlier paper (1891) he made a careful study of the chromatophore of the mature cell and its behaviour during swarmer formation. He points out that in this sheet gaps occur, the size of which depends on nutrition—in an undernourished net the gaps are large so that the chromatophore becomes net-like, while with ample food the net-like appearance is lost, since the gaps in the green cylinder become relatively narrow.

Timberlake (1901, p. 622) states that *Hydrodictyon* has no differentiated chromatophore, the chlorophyll being distributed generally in the cytoplasm and the net-like appearance being due to vacuolation.

Oltmanns (1922, p. 278) accepts Artari's view that the chromatophore is in the form of a mantle of the reticulate type such as is found in *Edogonium* and other algae.

Lowe and Lloyd (1927, p. 282) believe the young chloroplast (i.e. in the cell of a newly formed net) to be spiral in form "much as in the *Zygnemales*", later developing into a "network of small irregular chloroplasts frequently connected *inter se* by thin threads. The total arrangement is such however that the spiral structure can frequently be traced even in the mature coenocyte".

Fritsch (1935, p. 171) states that the cells of the net have a "complex reticulate chloroplast" but refers also to the work of Lowe and Lloyd.

Among the earlier workers the observations of Braun are outstanding, but in the latter part of his work quoted above he was obviously working with impoverished material since in well nourished young nets the chloroplast does not appear net-like. Artari and Klebs had the advantage of 40 years or more of progress in botanical science during which knowledge of cell structure and microtechnique were making great strides. They all agree in regarding the chlorophyll-bearing part of the mature cell as essentially a single structure which may become much divided so that it appears net-like. Klebs alone recognised that its form depends to a great extent on nutrition, that the formation of large gaps results from insufficient nourishment and on addition of fresh culture solution such gaps disappear. All describe the division of the chloroplast during the stages leading to the formation of reproductive bodies but show divergence of opinion as regards the breaking up of the chloroplast, Artari holding that the parts equal the nuclei in number, i.e. he holds that each motile cell has a single lobed chloroplast, whereas Braun and Klebs both describe a number of distinct rounded chlorophyll granules. Examination with the oil immersion lens shows that in the zooids and in most cases also in the gametes of both species, there are certainly a number of distinct rounded chlorophyll-containing bodies. On the other hand, in the swarmer of *H. patenaeforme* and more rarely in the gametes of *H. reticulatum* the chloroplast may sometimes appear to be undivided but lobed. No strands connecting the separate chloroplasts in either the pavement stage or the zooids could be detected.

Very different is the case in badly starved nets where breaking up of the pigmented areas has progressed so far that it has passed the stage of a net-like structure, however attenuated, and has the appearance of separate granules; here the granules are connected by colourless threads of varying thickness.

In the normal course of events the chromatophore of *Hydrodictyon* is highly polymorphic. In the well-nourished vegetative condition in the mature net it is essentially a continuous though perforated plate, and Timberlake's conception of its nature as chlorophyll distributed generally throughout the cytoplasm seems nearest the truth. Here the gaps in the pigmented region are small and linear and it is only in the impoverished net that they become so much enlarged that it appears reticulate. As the time for zooid or gamete formation approaches the perforations increase in size and number, become confluent, forming elongated slits which tend to arrange themselves in convoluted lines, and finally the whole chlorophyll-containing layer is broken up into small separate bodies each of

which contains a central proportionately large, or several smaller, starch centres and this is the condition throughout the motile stage. Immediately on return to the vegetative state the separate chloroplasts begin to coalesce and once again the continuous parietal plate is formed. Thus in deciding the form of the chloroplast two distinct factors have to be taken into consideration, one internal—the stage reached in development—the other external, i.e., the amount of available food. An examination of the figures given in the paper by Lowe and Lloyd and of the conclusions reached by these two workers forces one to deduce two facts: first, much of the material used showed marked signs of undernourishment (the photographs shown on Figs. 1, 4, 5 and 7 on Plate I and Plate IV, Figs. 4—10, are all of coenocytes exhibiting all the phenomena characteristic of partial starvation). The same is true in the case of the coenocyte shown in Plate III, Fig. 5; large vacuoles of the type shown, though of common occurrence in the “pavement” stage of coenocytes which have recently undergone a period of undernourishment are not seen in fully normal coenocytes; on the contrary, there the “pavement” is uninterrupted, lining the entire wall. Second, the distinction between an “adult” coenocyte and one which is preparing to divide is ignored, for instance, Plate I, Fig. 6, and Plate III, Fig. 3, show parts of coenocytes in which the pyrenoids have all but disappeared, while the nuclei show up beautifully, each surrounded by numerous starch-containing chloroplasts, the stage immediately preceding cleavage. As regards the spiral structure described by these workers, here again starvation has led to abnormal forms. Since the chloroplast formed by coalescence of the separate chlorophyll-containing bodies in the net-forming zooid does not completely encircle it, it follows that as the pigmented region extends, the two ends of the girdle do not always exactly meet and it may at first appear spiral in form, but if this is the case it is of short duration and soon the chloroplast extends in all directions until it completely lines the whole cell wall. A true spiral chloroplast such as is formed in *Spirogyra* is never seen in any normal coenocyte of average age.

The observations of Klebs on the behaviour of the chloroplast throughout the life cycle still remain unrivalled. But apparently he sometimes found complicated chloroplasts, one inside the other. Nothing of the kind has been seen in the present investigation nor can any explanation be suggested, except possibly an accidental infolding of the coenocyte. There has never been anything but a single parietal layer of chlorophyll-containing cytoplasm.

A comparison of cell structure in the three species shows that it is essentially similar in all; there are differences in detail somewhat difficult to

define; the chromatophore of *Hydrodictyon patenaeforme* appears to be rather more granular but with fewer perforations than that of *H. reticulatum*, while that of *H. africanum* is rather coarser grained than either of the other two, but in all, similar changes are brought about by variations in food supply, light, etc., and all undergo a similar sequence of changes during the life cycle although here too there appear to be slight differences more in the degree of any one change than in kind; for instance, in that stage of preparation for division characterized by the whorled pattern, the patterning in *H. patenaeforme* appears much finer than in *H. reticulatum* but, again, details of structure and appearance even in one and the same species vary so greatly under differing conditions that it is next to impossible to make any clear-cut distinctions. The figures given in Klebs' papers and reproduced by Oltmanns (1922, p. 278, Fig. 187), though somewhat diagrammatic, give a very good idea of the appearance of the chloroplast in the mature well-developed coenocyte and the changes therein during the preparation for division. The photographs shown here (Pl. XIV, XVII and XVIII), taken with the 1/12 inch oil-immersion lens, of living coenocytes on agar give a better picture of the actual appearance at the various stages, but here, as elsewhere, though these are typical there may be many variations in detail of appearance, depending mainly on external conditions. This is especially true as regards the proportion of fenestration (perforations or minute vacuoles, whichever term is preferred) in the chromatophore. As already shown this, although always present, varies in its extent depending directly on nutrition; undernourishment increases the relative size and extent of the non-pigmented areas, ample food supply reduces them until they are only discernible under high magnifications. It is as well to emphasize here that the term "reticulate" is only applicable to the chromatophore in the starved or partially starved condition. It is interesting that a partially starved net may undergo division without previously recovering fully from starvation; in such cases there may be extensive gaps in the protoplast alternating with areas showing normal "pavement" which eventually produce swarmers, or net-forming zooids as the case may be. This would account for many abnormal nets in the case of *H. reticulatum*.

A further point of interest which has apparently been generally overlooked is the change in the relative positions of nuclei and chloroplasts during zooid and swarmer formation. In the "pavement" stage the nucleus, as Braun had already noticed, occupies a central position surrounded, above and below as well as at the sides, by green plastids (Pl. XVIII, B). After completion of division however it changes its position, coming to lie immediately below the surface at one side instead of in the centre of the zooid (Pl. XVIII, C). Hence at this stage since the

colourless apex of each individual cell, containing the nucleus, lies at the side of the segment, the effect is of a separation between them although actual separation has not yet begun, and is very characteristic. It is even apparent at low magnifications but what then appear as spaces are shown by higher magnification to be actually the colourless portions of the developing zooids or swimmers.

9. THE PYRENOIDS.

The complete disappearance of the numerous pyrenoids as the coenocyte prepares to divide, already noted by Braun (1851. p. 280). is most characteristic of the genus. It accompanies the changes in the chromatophore preceding division and is usually complete when the pavement stage is formed: disappearance of the pyrenoids is accompanied by the appearance of many minute starch grains scattered throughout the cytoplasm. Most observers agree on the formation *de novo* of pyrenoids during the reconstruction of the chloroplast in the cell of the young net. Artari (1891. p. 281). it is true, states that when the "macrogonidia" round off preparatory to net formation in each one pyrenoid was always observed, but this was probably a slip and should read "one nucleus". since he had just described the disappearance of the pyrenoids.

Opinions differ however as to the origin of later formed pyrenoids. Braun (l.c., p. 211) believed that all were formed *de novo*; on the other hand Artari (l.c., p. 283) held that while the first one was formed *de novo*, subsequent pyrenoids arose by division of pre-existing ones; finally, Timberlake (l.c., p. 629) believed that some are formed *de novo*, others by division.

In the present investigation, no sign was seen of division of pyrenoids in the spores nor in the young cell. In the latter, the second pyrenoid usually arises far from the first, and the same is true where a second pyrenoid appears in a hypospore—quite often it is on the side farthest from the first and obviously, like the first, is formed *de novo*. In older coenocytes, however, cases of dividing pyrenoids have been observed. In one case in particular, where addition of fresh culture solution to a rather crowded culture had resulted in a sudden recrudescence, the coenocytes, already large, showed a number of very large pyrenoids which were deeply lobed and evidently in process of division, and in addition scattered among these and the numerous normal-sized pyrenoids (7—8 μ in diameter), all of which showed extensive starch sheaths, were numbers of minute pyrenoids (2—4 μ) evidently just formed. Hence the conclusion arrived at here is that in this genus the pyrenoid in general arise *de novo*, but may also be formed by division of pre-existing pyrenoids.

Timberlake (l.c., p. 619) studied pyrenoid structure and starch formation in fixed and sectioned material. A study of living material in different stages and at various times makes the acceptance of parts of his conclusions impossible. While his main thesis, namely that "the pyrenoid is directly the seat of the processes resulting in the formation of starch", must be accepted, his view that curved starch grains are cut off from the pyrenoid and *pushed outwards intact* to be distributed through the whole protoplast (p. 626) is quite untenable as immediately becomes apparent on examination of living cells.

In the vegetative state, that is, during the whole period of growth from the time when, directly after net formation, the chloroplast is reconstructed and the first pyrenoid appears until the preparations for division are well advanced, the entire starch content of the coenocyte is concentrated in the pyrenoids. During this period in a normal cell the chromatophore itself presents a remarkably homogeneous appearance between the perforations—clear green, without granules. Treated with iodine the pyrenoid alone gives the starch reaction, the starch sheath staining dark blue-black with a blue-green border as seen in optical section, the body greyish purple, probably owing to the enveloping starch sheath. The grains or slabs of starch enveloping it give it a polygonal outline and vary considerably in number and form. No sign of starch appears in the chromatophore itself; even when preparations for division have progressed as far as the "whorled" stage (Pl. XIV, D; XVII, C) stroma starch is still absent. But soon after, as the chloroplast divides into small portions, a faint starch reaction becomes apparent; in the smaller portions a single starch centre forms, in the larger segments two or three such centres appear. The starch reaction grows progressively stronger until finally, when division is complete, each small rounded chlorophyll-containing body has an accumulation of starch in its centre. This has not the appearance of definite grains—the iodine reaction is much what one gets in a colloidal solution of starch. Meanwhile the pyrenoids have completely disappeared and one is forced to the conclusion that their starch content has been transferred to the discrete chloroplasts. This transference is of course not one of entire starch grains; the pyrenoid starch is obviously altered to a soluble carbohydrate which is transformed again to starch in the chloroplast. Beyond the fact that enzyme action must obviously be involved, nothing is known as to the method of transfer. It seems as though the chloroplast changes its nature entirely and not only its outward form. In the vegetative phase it is the pyrenoid which is immediately concerned with starch formation, whereas with the disappearance of pyrenoids in the reproductive phase the chloroplast itself takes over the work of formation and storage of

starch, apparently functioning in much the same way as the chloroplasts of higher plants, but differing from them in that the starch is localized in the central region and not spread over the whole body of the chloroplast. Timberlake's Figs. 6 and 7 are probably taken from cells in the growing or vegetative state, while Fig. 1 is from a coenocyte already far advanced in preparation for division—the number and position of the nuclei alone indicate this. The structures he took to be starch grains are actually segments of the chloroplast, each with its own developing store of starch.

As regards the position of the pyrenoid relative to the chloroplast, Timberlake thought that "both pyrenoids and nuclei are . . . scattered throughout the protoplasmic layer". His Fig. 26 shows part of a coenocyte in which the chloroplast is already divided into discrete plastids which are surrounding the nuclei. In the living cell during the vegetative phase, the parietal chlorophyll-containing layer is thin and closely adpressed to the wall; in optical section (Fig. 5, d) nuclei and pyrenoids can be seen projecting from the inner surface of the chloroplast, the pyrenoids usually about half embedded, half projecting, the nuclei projecting almost entirely. As the reproductive stage approaches the chlorophyll-containing protoplast thickens considerably, the nuclei take up a central position with chlorophyll both inside and out, while pyrenoids, if any survive, still project, far more than in Timberlake's figure.

10. COMPARISON WITH THE VOLVOCALES.

As compared with the Volvocales, *Hydrodictyon* shows certain marked differences, in addition to those attributable to the coenocytic nature of the cell; of these the most suggestive concern the flagella and the pyrenoids.

In the higher Volvocales at any rate, the flagella are permanent structures, once formed they cannot be withdrawn, instead, when their work is finished they are either cast off, as happens in some species of *Carteria*, or remain attached to the cell wall but inactive, well seen in *Haematococcus* and in the Volvocaceae (*sensu* Smith) during formation of the germ colony. In *Hydrodictyon*, on the other hand, when the motile cell comes to rest preparatory to entering on the non-motile phase, it proceeds to withdraw its flagella which are generally absorbed until no trace of them remains. The method of this reabsorption was beautifully seen in the planozygotes of *H. reticulatum* described above (p. 74 and Fig. 6, f, g). Here the flagella seem to be merely specialized processes of the protoplast, whereas in the Volvocaceae they have to some extent the nature of permanent organelles.

The difference in the pyrenoids is less clear cut and more a matter of degree, but on the whole in the Volvocaceae the pyrenoid is a more permanent constituent of the cell, though even there preparations for division are often accompanied by the partial or complete disappearance of the pyrenoids. But nowhere is there the regular sequence of disappearance and subsequent reappearance of pyrenoids as found in *Hydrodictyon*. Further, the motile reproductive cell in the Volvocaceae, whether gamete or zoospore, normally contains one or more pyrenoids whereas in *Hydrodictyon* the motile cells are typically without pyrenoids. Finally, in the Volvocales formation of new pyrenoids by division of pre-existing ones is much more general than in *Hydrodictyon*.

11. STRUCTURE OF THE WALL AND VESICLE FORMATION.

Differentiation of a wall begins as soon as the motile cell settles down, whether as part of a net or as an independent body, be it a polyhedron or a hypnospore. At first the wall consists of a delicate membrane; later an inner laminated hyaline zone is formed while the outer membrane thickens slightly. Thus the wall is eventually two layered while, in addition to the outer cellulose wall in the spore and polyhedron, there is usually a very thin outer pectic layer. In the coenocyte the inner layer remains thin until the reproductive stage approaches and only then does it become obvious. In the hypnospore and polyhedron it develops earlier and in the latter the conical processes become more or less completely filled with an accumulation of inner-wall substance, thus forming firm spine-like structures which are no doubt protective against ingestion by small animal organisms. The polyhedral form is also, of course, adapted to a planktonic mode of life and helps to keep the developing cell above the substratum, while the pectic outer layer of the surface membrane causes it to adhere to aquatic plants or plant debris in the water.

In the mature net this outer mucilaginous layer appears to be absent, so that the net is not slimy although not harsh to the touch but silky, whence the description "*Musculus aquaticus bombycinus retiformis*" of Loeselius (1654, p. 51). As a consequence, the wall forms an excellent substratum for many plant and animal organisms. In the case of *H. patenaeforme*, *Stigeoclonium* sp. and a small species of *Coleochaete*, as well as many other algae, may grow on the older coenocytes.

A curious feature is the development of peg-like ingrowths from the wall into the protoplast of the coenocyte. These are formed of highly refractive hyaline substance, much laminated, and project inwards displacing the protoplast. Similar structures were described by Iyengar (1925) in *Hydrodictyon indicum*. In *H. patenaeforme* they are comparatively rare but in *H. reticulatum* they are often seen and are sometimes

complex (Fig. 5. j. and Pl. XVIII, B): they are also not uncommon in *H. africanum*. In *H. patenaeforme* they have occasionally been seen in separate coenocytes formed directly from polyhedra (Fig. 4, f).

With methylene blue the surface membrane stains deep purple, the inner layer when swollen up a paler purple with an almost unstained outer zone.

The alteration of the substance of the inner wall, which always accompanies the formation of motile cells, is intimately connected with the liberation of the latter: in each case the wall passes through a parallel series of changes brought about by the action of enzymes secreted by the increasingly active protoplast as it prepares for the motile phase and hence directly controlled by the protoplast. The first obvious change is gelatinization of the inner wall which begins to swell up, thus bringing to bear increasing pressure, outwards on the surface membrane and inwards on the plasma membrane, with varying results in the three cases, coenocyte, spore and polyhedron.

In the coenocyte the thin outer membrane exhibits marked elasticity and can undergo a considerable amount of stretching; when the limit of tensile strength is reached it splits, the method of splitting showing considerable variation. In net formation it often happens suddenly either near the centre of the coenocyte or near one end and the recoil throws the thin membrane into folds like a wrinkled glove (Pl. XII, A). In gamete formation, particularly in coenocytes in which increase in length is rapid, the outer membrane may split in a number of places; this may result entirely from pressure or possibly alteration in the outer membrane itself has taken place since the whole membrane behaves as if it were slatting, like perished silk. In all such cases observed, the central vacuole had remained intact and the whole structure inside the outer plasma membrane was in a state of turgor. Where however the central vacuole had collapsed, thus suddenly reducing the internal pressure, the wall substance thus released from internal pressure expanded inwards helping to force the swimmers out of the coenocyte: here the outer membrane remained intact except at the actual split through which the gametes were escaping (Pl. XIV, G). With their escape, secretion of enzymes stops and consequently changes in the wall cease. But if a daughter net is being formed, the zooids continue to secrete wall-modifying substances and gelatinization of the wall may continue long after net formation is complete, the remains of the inner wall forming a protective mucilaginous layer round the young net in the early stages of its development before ultimately disappearing entirely; such mucilaginous sheaths may be seen in Pl. XII, B—D. If the zooids, instead of forming a net, escape, again the reaction ceases. In this case and in the second type of gamete liberation the coeno-

cyte retains its form and may persist in the net for some time; since swarmers of whatever kind tend to collect on any available surface as they come to rest, such "ghost" nets may be outlined in green from the covering of young hypnospores on the walls of the empty coenocytes. Finally, if the zooids neither form a net nor escape but settle down within the coenocyte, again wall reaction ceases and the wall may persist for a long time.

The vesicle which is sometimes seen in gamete liberation is formed by the outer plasma membrane protruding through the split wall, forced out partly by pressure of the wall, partly by the gametes themselves. The gradual swelling of the vesicle thus formed may be due to osmotic activity of the swarming gametes but more probably is purely mechanical, caused by the gametes swarming out into it; in any case it soon disappears whether by complete gelatinization of its substance or by rupture due to pressure and the gametes are liberated into the water. Such vesicles have been observed only in *H. reticulatum*.

In the hypnospore the outer wall being thicker is more resistant and practically inextensible and a somewhat different procedure results. Some gelatinization takes place throughout the inner wall but is very much greater at one spot which will constitute the anterior pole of the developing spore (Fig. 1, g) and at which the exospore eventually ruptures; further changes cause the protrusion of the inner layers through this split, widening it until the orifice is nearly as wide as the diameter of the spore, and a vesicle is gradually formed (Fig. 1, h—k). The vesicle here is a much more significant structure than that described above, and its behaviour is less easily explained. Its formation accompanies the passage outward of the protoplast which however is not in contact with it. Its enlargement is therefore not the result of direct pressure. Moreover, treated with methylene blue the wall of the spore stains much as does that of the coenocyte—outer membrane deep purple, inner pale purple, but the wall of the vesicle remains unstained. The mode of formation suggests an osmotically active structure, the wall of which is extensible and therefore increases in size as internal pressure rises. The vesicle wall is probably formed by the plasma membrane supplemented by part of the wall substance and is thus a semipermeable membrane. The developing zoospores produce osmotically active substances as they pass out into the vesicle, which constantly enlarges until finally the limit of tensile strength is reached and it ruptures distally; as the internal pressure is released the mucilaginous layers of the spore wall expand inwards into the empty spore.

There is seldom much flagellary action within the vesicle; the flagella can often be seen moving with a curiously undulatory action, without

imparting movement to the zoospores, particularly if the spore is azygotic and contains only a single zoospore. The greatest activity seen occurred in some very large zygosporangia of *H. patenaeforme* where the eight zoospores actually moved about actively within the large vesicle, but this was exceptional and normally the zoospore or spores remain apparently passive; when increasing pressure ruptures the vesicle they may be shot out with considerable force and even when, as in the case of the eight zoospores described above, escape has been preceded by active movement, during the actual moment of expulsion the zoospore may be quite passive as it passes out of the vesicle, and remain so momentarily after it is freed, before becoming actively motile and swimming away. In this connection the case of those azygosporangia of *H. patenaeforme* in which the orientation of the single large zoospore was reversed and the zoospore consequently impelled backwards out of the ruptured vesicle is significant.

Finally in the polyhedron yet a third type of procedure is found. The contents are very dense; the ratio of the volume of the protoplast to that of the central vacuole is far higher than in the coenocyte, hence although the germ net is much smaller than the daughter net, there must be a far greater expansion in proportion to the size to provide sufficient room for the net to form properly while yet being adequately protected. As in the spore the need is met by the formation of a vesicle but here formed rather differently. Provision for the additional material needed in the formation of the proportionately larger vesicle is made by the accumulation of inner wall substance within the conical spines; between the spines the wall remains relatively thin but when mature the spines are almost solid wall substance, the outer membrane being continuous over the whole structure. Thus in normal well-formed polyhedra the protoplast remains approximately spherical, projecting only slightly into the base of the spines. If the polyhedron matures rapidly the outer membrane is apparently little altered and on germination may contribute to the vesicle wall. Where however maturation is slower, the outer wall is correspondingly toughened and only the inner layers are gelatinized to form the vesicle wall, the outer membrane being sloughed off. In *H. patenaeforme* the whole wall may thus contribute to the formation of the vesicle (cf. Pocock, 1937) but more usually only the inner wall, spines included, provides the reservoir of material needed for the vesicle wall. In *H. reticulatum* in none of the cases where germination was watched was the outer skin thus utilized, but in a number of the already formed germ nets, the vesicle instead of being rounded was angular, retaining the shape of the polyhedron. It seems probable that in such cases the outer membrane has contributed to the vesicle wall (Pl. XIX, L: Mainx. p. 513 and Fig. 15). Here, as in the spore, the plasma membrane probably helps in the forma-

tion of the wall since the gradual expansion, which begins with the first movement of the zooids, accompanies the process of net formation and continues after the net is formed, suggests that osmotically active substances are being secreted by the zooids and that here, as in the spore vesicle, the wall of the vesicle is a semi-permeable membrane.

Since changes in the wall are governed by secretions of the protoplast, conditions interfering with the normal metabolism of the latter will result in interference in the sequence of changes. Here, as in daughter-net formation, incomplete gelatinization of the wall results in a crowded, ill-formed net, with many random unions between the component cells. In germinating spores the reactions responsible for the formation and rupture of the vesicle not infrequently fail or are only partially successful; in the first case polyhedra may be formed inside or half inside the spore coat, in the second, inside the vesicle (Fig. 1, q). Eventually growth of the polyhedron ruptures the spore or vesicle wall (cf. Pringsheim, Figs. 10, 11). In crowded or contaminated cultures of polyhedra the vesicle may fail to develop properly and consequently the net cannot form normally. Young very congested nets are sometimes found within the partly expanded polyhedron wall, or the zooids form an amorphous mass. Pl. XIX, J, shows a polyhedron of *H. reticulatum* in which development of the vesicle began normally but was arrested before nearly complete so that there was not room for the zooids to separate and rearrange themselves in net formation. Such abnormalities are probably due to some deficiency in the zooids themselves, which are consequently unable to secrete the necessary enzymes in sufficient quantities to bring about the formation of the normal vesicle, the lumen of which consequently remains too small to provide the space necessary for net formation; this reacts again on the crowded zooids which presumably cease prematurely the production of the necessary enzymes.

The whole question of the production and control of substances concerned in such vital processes is a fascinating one. Among algae it appears to be at present an almost untouched field. The presence of a "growth substance" has been demonstrated in *Valonia macrophysa* by Van der Weij (1933, a, b) but otherwise little or nothing has been done towards solving this and many other biophysical problems in this group. *Hydrodictyon* would seem to offer fine opportunities for research along such lines, particularly in view of the ease with which it can be raised in culture.

12. PROVISION OF SPACE FOR MOVEMENT OF ZOOIDS AND SWARMERS.

How the space necessary to allow of the slight separation of either zooids or swarmers before movement can begin is provided, presents yet

another problem. The accepted view is summed up by Fritsch (l.c., p. 172) thus: "the necessary space for the movement of the swimmers is created by the swelling of the longitudinal walls and a reduction in the size of the vacuole". This was the view held by Jost, whose material however appears to have been somewhat abnormal. Klebs (1891, p. 17) describes an expansion of the wall and a simultaneous shrinkage creating gaps in the protoplast due to plasmolysis: "the vacuolar membrane remains tense until the end of zoospore formation" and, in addition, the individual spores themselves, he believes, are plasmolyzed and thus decrease in size.

Shrinkage of the vacuole, to which according to both Klebs and Jost the developing spores adhere, would merely have the effect of bringing them closer together. It is possible that there is a slight shrinkage in the individual parts of the protoplast as they are transformed into motile cells, but measurements taken in the pavement stage and of the zooids (or gametes as the case may be) as they begin to move are not conclusive; so far as could be judged, there was no appreciable decrease in size and the question therefore arose as to whether there were actually a shrinkage of the protoplast as a whole. Measurements were made at intervals of a few minutes of the external diameter of the coenocytes and the diameter of the protoplast as seen in optical section at three spots, near the two ends and in the centre. In addition, in some cases the length of the whole coenocyte was also measured. The results showed that, in net formation, there is a gradual increase in the external diameter of the coenocyte and also, but slightly less, in the diameter of the protoplast; further, the whole coenocyte increases in length to a varying extent; thus while the wall is increasing in thickness, through the gelatinization of the inner layers, the whole surface area of the coenocyte is increasing, thus tending to separate the individual parts just sufficiently to allow of initiation of movement. As net formation begins, the efforts of the individual zooids serve to increase the space between them and consequently the size of the whole continues to increase throughout net formation. Eventually the rupture of the external wall is marked by increase in the swelling of the now unconfined mucilaginous inner wall.

In the case of gamete formation the initial stages are the same, i.e. both wall and protoplast increase in diameter, but once movement has started not only does the wall stop enlarging but there is often a marked decrease in the diameter of the protoplast. In other cases, however, the protoplast continues to enlarge, even more so than in the case of net formation.

It must however be emphasized that this description of the course of events refers only to the fully normal coenocyte, that is, one in which

in the pavement stage there are no gaps in the protoplast. If, on the other hand, the protoplast is extensively vacuolated, as is the case when under nourishment has resulted in a reticulate chloroplast, the necessary space is obtained by a filling up of the gaps—the zooids adjacent to a gap can be watched moving inwards into it—and thus gradually the whole mass is loosened sufficiently to allow of general movement. There is obviously a close relation between the degree of vacuolation and the amount of increase in size needed.

In those cases where, instead of uniting, the movement of the zooids strengthens and they finally escape, the initial increase in size of the protoplast stops and the procedure is reversed, the central vacuole decreases, the membrane sometimes disappearing altogether, and swarming takes place as in the case of the gametes. As in the alteration of the wall, the zooids are here producing substances which react on the central vacuole. In both this case and in gamete formation, since the reaction ceases early, the outer wall remains intact, except at the point of egress.

13. MECHANISM OF NET FORMATION.

The peculiar, restricted type of movement exhibited by the zooids during net formation has impressed all observers of this phenomenon. Areschoug (1842, p. 130), one of the first who actually watched net formation, compares the movement of the zooids to molecular movement and the comparison is apt. But owing in part to its brief duration, in part to difficulties of observation (among which not the least is the untimely hour—dawn or soon after—most often chosen by the organism for this particular stage in its life history), an adequate explanation of its nature has not been forthcoming. Klebs, in his earlier paper (1891, p. 789), gives a detailed account of daughter-net formation, in which he described the zooids as connected throughout by protoplasmic strands, and since the same statement is repeated in his later work (1896, p. 135) he apparently saw no reason to doubt his original observation. He particularly noted the persistence of the vacuolar membrane which “remains tense until the end of zoospore formation” and lays stress on the changes in the osmotic pressure of the cell sap, holding that plasmolysis, both of the cell as a whole and of the individual segments of the protoplast, plays important rôles at certain stages of the process. Further, he believed that to begin with the zooids were actually attached to the vacuolar membrane by fine protoplasmic threads (1891, p. 854) and remained thus to the end of the process, their movement being confined to a quivering: “sie zittern auf der Stelle hin und her”. He thus ascribes to the vacuolar membrane a most important controlling rôle in the process of net formation, but in

his later work (1896, p. 155) he does not even mention its existence and thus seems to have changed his mind as to its importance.

Harper (1908) found no connecting strands between the zooids: on the contrary, he believed they moved freely among themselves, but his account of net formation suggests that he was actually describing two different processes, since in net formation the zooids never move into the centre of the coenocyte and then swim back to the outer wall, as he describes, nor is their movement ever very strong. He was probably misled by observing coenocytes in which swarming was in progress and thus his account of the mechanism of net formation is of little value. He apparently did not observe the vacuolar membrane and no further reference to this structure is found in the literature until Jost (1930, p. 61) again described it.

Jost agrees with Harper as to the absence of connections between the zooids during the formation of the net, although he does not deny that they may be present at some stages; but, if so, he did not believe that they ever had the significance Klebs attributed to them. He evidently believed that the vacuolar membrane plays an important part in net formation and noted that it remained intact for an extraordinarily long time. He found that it was possible to plasmolyze the coenocyte even after movement of the zooids had begun, but he is not clear as to how it acts, and from his description of the presence of large gaps in the protoplast and extreme shrinkage of the vacuole with zooids adhering to it, it is probable that his material was not normal. Similar behaviour has been observed in the course of this investigation in cultures which had been partially starved. He lays stress on the adherence of the zooids to the vacuolar membrane "as if they were connected to it or in some way held on the surface".

More recently, L. Moewus (1948) has recorded the existence of fine protoplasmic threads actually attaching the zooids to the vacuolar membrane.

In the present investigation nothing has been found in support of this view. On the contrary, all the evidence indicates that the zooids move freely within the parietal space enclosed between the protoplasmic and vacuolar membranes but are confined within these two membranes; since the distance between them is only a little greater than the diameter of the zooids, movement in a radial direction is strictly limited, so that the zooids must form a single layer parallel to the wall of the coenocyte. Thus the importance of the vacuolar membrane in regulating the form of the net can hardly be over estimated.

In early stages of differentiation of the zooids it was sometimes possible to see connecting strands between them (cf. Fig. 5. 1). It seems probable

that when cytoplasmic cleavage takes place, such strands may remain only to disappear completely as development proceeds and the zooids separate. When movement is at its height, zooids can be seen turning and twisting completely round and even changing their position relative to their neighbours. It is therefore obvious that, in general, they are completely free from one another. It is however also obvious that some restriction to the free movement of the zooids exists. The enlargement of wall and protoplast and changes in shape of the individual zooids provides the space necessary for initiation of movement; the space is gradually increased largely by the activity of the zooids themselves, but all such movement is in one plane. Movement outwards is restricted by the cell wall with its protoplasmic lining, movement inwards by the wall of the central vacuole. The close proximity of the zooids to one another no doubt contributes to their readiness to unite with one another.

Should the vacuolar membrane contract markedly or not persist, there is nothing to prevent free movement towards the centre of the coenocyte, the incipient motion characteristic of the zooids in net formation strengthens and the zooids behave like swimmers; it is probably some such change in the membrane which determines whether the zooids shall function as free swimmers instead of forming a net.

What factors cause the disappearance, or even a great diminution in size, of the central vacuole is not known, but since its persistence or disappearance probably depends on some enzyme action it may well be that the reason for its non-persistence must be sought in the zooids themselves, that in them the net-forming urge is in some cases subordinate to the urge to move actively, that their greater activity is accompanied by a difference in enzyme secretion which affects the vacuolar membrane.

Once net formation has started the urge to unite may be strong enough to persist, even when the confining agents have disappeared as was the case in the examples of coenocytes torn or otherwise damaged when in the process of net formation already described—the zooids even uniting to form portions of net when already free in the water.

Sometimes however zooids liberated in this way settled down and formed hypnospores while yet others formed isolated coenocytes. Which of the three alternatives any particular zooid will follow, depends on the stage reached by it when liberation took place.

In the case of the germ net the method by which the vacuolar membrane functions is not so obvious. Probably its main function here ends with the initiation of net formation during which it helps to disperse the zooids and prevent random unions in different planes. It is always very much smaller than the vesicle at the close of net formation and may

disappear early in the process, or persist even after the germ net is formed,

In *H. patenaeforme* and *H. africanum*, since the germ net normally flattens out completely, it is difficult to see how the vacuolar membrane can exercise any controlling influence except in the earliest stages when the mass of zooids is opening out, yet even here it may persist and can sometimes be seen to one side of the net as a tiny spherical bubble. In the sac-shaped germ nets most often formed in *H. reticulatum*, however, its function may continue throughout net formation, the net resulting from an early union of the zooids while still in the peripheral position of their origin. Congested tangled nets such as may sometimes occur, no doubt result from the premature collapse or disappearance of the vacuole.

In germ nets, too, instances of net formation continuing free in the water have been observed, sometimes traumatic in their origin, sometimes resulting from premature disintegration of the vesicle. Here again the liberated zooids may form a net, or hypnospores, or isolated coenocytes.

In certain abnormal cultures in which in addition to partial starvation phenomena, in particular extensive vacuolation, other unusual features were present, net formation was much on the lines indicated by Jost—the zooids formed groups separated by wide gaps and the wall swelled very much more than usual and unevenly, one end being very much wider than the other. As a result the zooids were left adhering to the central vacuole which, being proportionately so much smaller than the wall, *appeared* to have contracted. Whether there had actually been much contraction was doubtful, and the adherence of the layer of zooids to the membrane seems a natural consequence of the abnormal swelling and resultant displacement of the wall and does not necessitate postulating any actual union between zooids and membrane to explain it—the protoplast merely remains in its normal position. While clearly abnormal, the nets formed in this culture were yet of great value since the sparseness of the zooids and the irregular swelling of the wall made it easy to distinguish the parts of the vacuolar membrane, at first in position and later as a persistent sac, like a sausage balloon in shape. In normal cultures the membrane is seldom distinguishable after net formation is complete, but that it, as well as the plasma membrane, persists for some time after completion of net formation, in some cases at any rate, became obvious on treating with salt solution. Day-old nets thus treated remained unaltered in shape and size, although the individual coenocytes plasmolyzed, but newly formed nets in which although the surface membrane of the wall had ruptured, the inner layers still formed an unbroken sac round the net, contracted suddenly, then recovered their original form. Presumably the net, adhering to the vacuolar membrane, still behaved as a continuous protoplasmic layer. Jost mentions that

plasmolysis is possible during actual net formation; in some cases it is thus possible even longer.

At ordinary magnifications it is difficult to see the flagella of the zooids. Braun states that they appear to be very short: "Flimmerfäden . . . welche bei den Netz-bildenden Macrogonidien sehr kurz zu sein scheinen" and Cohn (1853) was unable to determine their number with certainty. Artari (l.c., p. 281), however, fixing with osmic acid or iodine and staining with gentian violet, was able to demonstrate the existence of two flagella shorter than or as long as the body of the zooid. Klebs (1896, p. 134, Fig. 4, C) shows them as equal to body length.

By using the 1/12 inch oil-immersion lens it is possible to see the flagella in the living state, while in iodine-fixed material they show up much more clearly. Usually they are short, less than body length, but here and there a zooid with long flagella may occur. When the zooids unite, the flagella are withdrawn in the same way as in the planozygote. If, on the other hand, instead of uniting the zooids become increasingly active and eventually swarm, the flagella elongate. During net formation the movement is such as one would expect from cells in which the flagella are not fully developed.

Reunion of the Zooids. The procedure followed by the zooids as they unite is most interesting and fascinating to watch. Klebs postulated a shortening of the connecting strands bringing adjacent zooids into close contact. Jost (l.c., p. 63), on theoretical grounds, holds that such strands cannot exist in net formation and his observations confirmed the conclusion so reached. Later, however (p. 69), when discussing the reuniting of the zooids he remarks "one might think that protoplasmic connections form between the cells of the net. I have found no evidence whatever for their existence". The movement of the zooids and the way in which they touch one another strongly recalls the efforts of conjugating gametes to effect contact, except of course that here contact is being made simultaneously at two opposite points at each of which in general the zooid unites with two other individuals. Usually the zooids are so crowded that the actual spaces between them are extremely narrow and it is difficult to see what really happens. Each zooid widens so that the equatorial diameter is greater than the polar, and union with its neighbours takes place laterally, the clear apex remaining free in the centre of one side of the mesh, facing into a space (Pl. XVIII, E). Sometimes a protoplasmic bridge seems to be present, at others union appears to be effected over the whole contact surface, while in less crowded conditions, such as obtain in germ-net formation, it is possible to distinguish actual protoplasmic strands already uniting zooids or thrown out by a zooid in its efforts to make contact with another. From the grouping in

the net it is evident that normally two such "feelers" or groups of feelers are put out at each pole of union, the tendency being for three cells to unite at each angle. This throwing out of processes by net-forming zooids is comparable with the similar phenomena which occur when a zoospore comes to rest and begins to form a polyhedron. The projections seen on the coenocytes at the edge of the germ net in *Hydrodictyon patenaeforme* and to a lesser degree in *H. reticulatum*, are obviously unsatisfied protoplasmic processes of this nature, i.e. processes which have failed to make contact with neighbouring cells but have persisted after the zooids have developed walls. In the photograph of a newly formed net reproduced on Pl. XIX, E, it is just possible to detect here and there connecting strands between the zooids. Union at this stage is very light, contact has been effected but union along the common surface is not complete and no walls have been differentiated, each zooid is still merely enclosed in the surface membrane of its protoplast. At this stage, very slight pressure on the cover slip sufficed to disrupt the newly formed net, in parts of it separating the cells completely.

With wall formation, the secretion of the common end walls makes union very much stronger and in the process all trace of protoplasmic connections between adjacent cells disappears.

14. "PERIPLASM" AND MONSTROUS CELLS.

Oltmanns (l.c., p. 280) speaks of plasm material or "Periplasm" left over in zooid formation. This does not usually happen: as a rule the whole protoplast, except the inner and outer plasma membranes, divides into zooids (or gametes) but occasionally a small part is left over. Where this happens it is always in coenocytes which are not quite normal and such cases would appear to be always pathological. Again, in fully normal coenocytes, division is markedly uniform and where zooids or swarmers of varying sizes are formed in one and the same coenocyte, as often happens in cultural conditions, they are abnormal and probably due to something which has occurred to upset the usual course of development. Monstrous cells, two to four times the diameter of the normal cell, have been observed in both net and gamete formation. They may be motile and may behave in every respect like their sister cells, uniting in the net, or if gametic, escaping with the rest. Such a cell may have one or several sets of flagellary apparatus.

In gamete formation one coenocyte may often produce swarmers of two sizes, one much larger than the other. Here when conjugation takes place, size appears of no importance in determining union—the gametes of a pair may be large and small or may be of the same size. This applies

to both *H. patenaeforme* and *H. reticulatum*. In *H. africanum* the anisogamy is of an entirely different category, and is normal.

15. ISOLATED COENOCYTES.

Jost (l.c., p. 7) obtained isolated coenocytes and figures a number, either separate or in small groups (Figs. 12—14). He watched the early stages of development but did not succeed in bringing them to maturity, probably owing as he himself suggests, to adverse cultural conditions. (From other internal evidence in his paper it would appear that the nutrient fluid he used was far from ideal.)

During the present investigation isolated coenocytes have been repeatedly obtained, formed directly from swimmers, from zoospores produced on germination of hypnospores, from zygotes and polyhedra, and from germ-net zooids in both *H. reticulatum* and *H. patenaeforme*, and in the former from daughter-net-forming zooids which, for one reason or another, have been prevented from uniting. However formed, such coenocytes develop normally in the nutrient solution used or on agar. Those from net-forming zooids usually take approximately the form they would have had in the net, becoming more or less elongated; those formed from other types of motile cells are usually more or less rounded, while those from polyhedra sometimes show some resemblance to the latter, but no matter what the origin or form, the development is essentially normal, those of *H. patenaeforme* eventually producing swimmers, while in *H. reticulatum* they may produce either swimmers or more usually daughter net.

16. CONJUGATION OF GAMETES.

Of the various observations hitherto published of the sexual cells, those of Mainx (1931) are the fullest. A further instance of the extreme plasticity of *Hydrodictyon* is afforded by the fact that in the course of the present experiments with *H. reticulatum* yet other variations in the behaviour of the gametes have been observed. Mainx (p. 504) found that the period of motility varied from 5 to 48 hours, during which time the gametes were at first strongly phototactic, then becoming negatively phototactic began to swarm, swarming being accompanied by marked group formation. Fusion began at the anterior end close to the insertion of the flagella and zygotes at once lost motility and surrounded themselves with a wall. Unsatisfied gametes which were unable to find a partner settled down similarly and secreted a wall, but did not develop further. Mainx particularly studied group formation and its connection with copulation.

Here there has been a difference in behaviour according to the season; in winter and spring the swimmers on the whole behaved much as did

those in Mainx's cultures. They showed marked phototactism, often remained motile for many hours and tended to clump. But in midsummer (December 1940 to January 1941) very different behaviour was the rule—the eyespot was poorly developed, phototactism not at all marked and the period of motility extremely short, often only a few minutes, copulation frequently taking place extensively even within the coenocyte wall after liberation of gametes had commenced. The liberated gametes tended to hang together in a single great crowd and did not form separate groups: the urge to copulate was evidently so strong that it overcame all external stimuli, including that of light.

A further difference was noted in the behaviour of the planozygotes. Unlike those observed by Mainx, here the planozygote never lost its motility immediately but always had a motile period during which its movements were as active as those of the gametes. This motile stage varied in length, apparently to some extent in proportion to the time which elapsed before conjugation. In the later cultures where conjugation was rapid, the planozygotes, easily distinguished from the gametes by their greater size, came to rest rapidly: usually within a few minutes of their formation they began to settle down, attaching themselves to some solid object such as the wall of a coenocyte, other spores already formed, etc. A short period of vacillation usually followed, marked at first by changes of position, then by jerking movements from the attached tips of the flagella, after which withdrawal of the flagella and gradual rounding off of the body followed, the whole completed within a few minutes.

Here again there is a difference in their behaviour from that described by Mainx—the indifference to light-stimulus exhibited by the gametes in these cases was shared by the planozygotes, which instead of collecting towards the light showed a strong tendency to attach themselves to any object in the water; in several cases what appeared to be an intact green net on closer examination proved to be one in which all, or nearly all, the coenocytes were empty, having liberated gametes, but the walls of which were thickly coated with green hyphospores already beginning to enlarge. Conjugation had probably begun as the gametes were liberated so that the planozygotes had formed in the immediate vicinity of the net which thus provided the nearest convenient surface to which they could attach themselves.

17. INITIATION OF GERMINATION, PREPARATION FOR DIVISION, ETC.

The initiation of the changes which lead to the formation of the motile phase at any point often seems to depend on some sudden change in external conditions, this change acting as a trigger mechanism. Thus in

the mature coenocyte of *H. reticulatum* addition of fresh culture solution will often precipitate daughter-net formation, whereas if left undisturbed development may continue for a long time without change. Similarly, when division has been completed there may be a more or less prolonged pause and the coming of daylight apparently serves to initiate movement of the zooids.

This trigger action is perhaps best studied in the hypnospor. In South Africa, where *H. patenaeforme* occurs in temporary rain-water pools and vleis, the releasing agent is probably often the sudden access of fresh rain water. As yet, however, little is known as to the development of the spores in the field or as to their size when in the resting state in the soil of the vlei bottom. Possibly direct sunlight may also act in some cases as the trigger. In culture, transference of mature spores from agar to culture solution sometimes at once initiated germination. In other cases transference from culture solution to distilled water and then back to dilute culture solution (usually 50—75 per cent) brought about germination, while in yet others the change had to be made several times before the "trigger" worked. Change in temperature also seems effective at times; which factor is the determining one depends to a great extent on the season of the year, that is, in all probability chiefly on the temperature. Since the first stage in germination must be the secretion of enzymes to bring about the necessary changes in the wall, the protoplast must be stimulated to begin activity in enzyme secretion.

18. FORM OF THE PRIMARY OR GERM NET.

Pringsheim (1861, p. 10), describing the development of the germ net from the polyhedron, says the "swarm spores" often form a single layered or almost single layered net, but more usually, as in the larger polyhedra with their richer cell content, there is a larger number of swarm spores a net, like the well-known net of *Hydrodictyon*, a perfect hollow sac is formed, and in his opinion this is the normal form. He gives two figures, one composed of few cells and in form an imperfect sac, the other of many more cells in the form of a perfect hollow sac. The latter, he states, is the normal form. Unfortunately all subsequent writers of algological textbooks, including Oltmanns and Fritsch, reproduce the first figure and ignore the one which Pringsheim explicitly states is the normal form of the net in *H. reticulatum*.

Mainx (l.c., p. 513), on the other hand, says "The primary net . . . is in contrast with the sac-shaped nets later formed by zoospore formation always formed in one plane."

In all the species of *Hydrodictyon* there seems to be a tendency for the mass of zooids forming the germ net to flatten out, the degree of

flattening reached before movement ceases, depending partly on external conditions, partly on the number of zooids concerned. The size of the vesicle is also important in determining the form of the net. If the number of zooids is great, as in very large polyhedra, the marginal zooids may have already made contact with one another and come to rest while there is still a more or less extensively crowded central zone. Hence there is not sufficient space for the zooids to arrange themselves on one plane and consequently they unite in all directions, so that the central region of the net may be from two to several layers thick. Flat nets are the general rule in *H. patenaeforme* and *H. africanum*: in both species many hundreds of germ nets formed in the field have been examined and the great majority were completely single layered. In culture of *H. patenaeforme* the number of nets with double centres was higher, and in badly contaminated or otherwise not perfectly healthy cultures tangled nets without definite form sometimes occurred. In general, the less crowded and more healthy the culture, the higher the proportion of flat nets. In some cultures, on the other hand, separation had proceeded farther than usual, resulting in a crop of flat nets each with a large central gap. *H. africanum* also sometimes showed double-centred nets when raised in culture, but more rarely.

In *H. reticulatum* the tendency for the germ-net zooids to arrange themselves in a single plane is very much less marked and often the ball of zooids simply opens out, forming a hollow net such as Pringsheim described. In such cases, apparently, union takes place rapidly before the vesicle had much enlarged so that the space between the vesicle wall and the vacuolar membrane remains comparatively small. A group of such nets is shown in the photograph reproduced as Pl. XIX, M; here the vesicles were persistent and many still showed traces of the form of the parent polyhedron, showing that vesicle formation had not been so complete as is usually the case in *H. patenaeforme*, and in a number the vacuolar membrane was still intact. Even so, completely closed nets were comparatively rare, though some nearly perfect "hollow sacs" were obtained (Pl. XIX, J—M); many were however open at one point and every degree of flattening was seen, some nets were bowl shaped, some basket shaped, others flattened ellipsoids while in many cross-unions had occurred, possibly due to the early disappearance of the central vacuole; on the other hand, the latter may persist even when the net is flat, if the vesicle has enlarged rapidly and to a great size. Of the nets raised in culture a fair number were flat but usually with a more or less extensive central zone of two or more layers; the single-layered border in these nets varied greatly in extent. A very large number were of the closed type but with cross unions, while tangled nets without definite form were

common in some cultures, usually rather crowded. Perfectly flat nets entirely in one plane, such as Mainx figured, were extremely rare.

Thus, even allowing for abnormal culture conditions—here as in the other two species tangled nets are almost certainly pathological—*H. reticulatum* shows a distinct difference in regard to the form of the germ net. In both *H. patenaeforme* and *H. africanum* the germ net is the only one in the life cycle and is typically flat and single layered, the number of zooids forming it being usually either in the neighbourhood of 512 (2^9) or 1,024 (2^{10}), but sometimes greater, and growth continues till it reaches a large size. In *H. reticulatum*, on the other hand, the germ net is, as it were, a temporary structure and is soon replaced by secondary daughter nets which assume the form of the coenocytes in which they are formed, and are thus in general closed cylinders; but even the germ nets in this species show a strong tendency to assume the form of a closed sac-like structure. Here the number of zooids is typically lower than in the other two species.

It is possible that the strains used by Mainx in his work showed a tendency to the flat form of net, or his culture conditions were such as to favour that form. The net he figures is composed of far fewer coenocytes (128) than was usually the case in the nets raised in these cultures. In view of the wide distribution of *H. patenaeforme* it is even possible that this species may occur in the warmer regions of Europe and that Mainx was dealing with a mixture of the two species.

In all species the marginal coenocytes show the horn-like outgrowths typical of cases where less than three coenocytes have united.

19. REGULARITY OF THE NET.

In *H. reticulatum* the life cycle is dominated by the secondarily formed daughter nets; not only are they far more numerous than the primary nets, but they are far more striking and more easily observed, so that from the first the whole conception of the genus has been founded on their characteristic form—a cylinder with closed ends. If the net is a normal, well nourished one its meshes are formed by cylindrical coenocytes and when it reaches maturity in general each coenocyte produces a daughter net; the layer of polygonal segments is continuous, lining the whole wall. When they are about to become flagellated zooids all are still in contact and by mutual pressure more or less hexagonal in surface view. During movement the whole mass gradually expands but mutual pressure still limits the possible distance between zooids, while flagellary action tends to create the necessary space. The result is a net remarkable for its regularity; despite a certain amount of variation in detail the

majority of the zooids are accurately united each with two of its fellows at the opposite ends of an equatorial axis, and the majority of the meshes so formed are hexagonal, although many may be pentagonal,—curiously enough both Ray (1686, p. 1852) and Areschoug (l.c., p. 128, and Plate V, Fig. 7) emphasize the "pentagonal mesh"—while others are formed by seven, four or even three cells. Nevertheless, owing to the uniform size of the cells, the general effect is one of very beautiful regularity. It is only when conditions are unfavourable, resulting on the one hand in an openly reticulate chloroplast leading to a diffuse net, or on the other in irregular congested nets in which failure of the due expansion of the wall results in abnormal crowding of the zooids, that marked irregularities become prominent. Instances of the former are described by Lowe and Lloyd (1927, p. 284, and Plate II, Fig. 5) who thought that this condition was the normal one and that the abnormalities they noticed in better nourished cultures resulted from "crowding of the zoospores", whereas such abnormalities result it is true in one sense from overcrowding, but due to feeble net formation in unfavourable culture conditions which do not permit of the normal sequence in net formation—probably the enzyme production of the zooids is enfeebled so that the wall is not sufficiently altered to provide the space necessary for successful rearrangement of the zooids *inter se*. Jost (1930, p. 61) likewise appears to have worked with undernourished nets since he states that at the first swelling of the cell wall the spore masses separated widely.

20. SEASONAL VARIATIONS.

The majority of the cultures of *H. reticulatum* dealt with in this work were made in winter and spring and, as already stated, these were marked by zooid formation, the zooids either uniting to form daughter nets or occasionally being liberated and swarming like gametes. Gamete formation, on the other hand, was rare and when it occurred conjugation was delayed. Hypnospores developed well and rapidly, particularly when kept in a warm room, and a little direct sunlight proved beneficial. Germination experiments succeeded readily in both *H. reticulatum* and *H. patenaeforme*.

When cultures were made in full summer, however, several differences became apparent. The nets still developed well and grew readily, but in *H. reticulatum*, though daughter nets were formed freely, liberation of zooids was rare. Gamete formation however was of common occurrence, the gametes showed little or no response to light but conjugated immediately on liberation. The spores developed well to begin with but later the cultures became badly contaminated and many spores were

destroyed by a parasite, possibly of the nature of a *Vampyrella*. When germination experiments were attempted, the hypnospores showed great reluctance to germinate. These differences are suggestive and it seems possible that the smaller difference between day and night temperatures may exercise a more important influence than the actual maximum temperature. A much fuller investigation is needed.

HISTORICAL AND TOPOGRAPHICAL NOTES.

Early references to *Hydrodictyon* are listed by Braun (1855) and need not be repeated here; but throughout the literature of this alga there is curiously little notice taken of one of the earliest records, that given in Ray's *Historia* (1686, p. 1852); although Morison (1683, p. 644) a few years earlier had included a description "*Musculus aquaticus bombycinus retiformis*" and figure of *Hydrodictyon*, it is to Ray, or rather to his collaborator, Samuel Doody, that the present specific name is due. Linnaeus (1753, V. 2, p. 1165), under *Conferva reticulata*, quotes as author Dillenius, and for *Conferva reticulata crispa* Plukenet, but does not mention Ray. Lamarck (1786, p. 81), under *Conferva reticulata*, quotes first Dillenius and then Ray. Both Plukenet and Dillenius however refer to Doody's record. Plukenet (1691, T. XXLV, 2) figures "*Conferva reticulata* Doody, Ray Hist. app.", while Dillenius (1741, Tab. LV, 14 A and B) figures "*Conferva reticulata* Doody. The Net Conferva".

Apparently Linnaeus had not seen the original reference in the *Historia* under the title "*3. Conferva reticulata* D. Doody". The brief but accurate description given by Ray is ascribed by him to Doody who had found it in ditches near Westminster and in streams on Hounslow Heath, tangled in the stems and leaves of plants: "In fossis prope Westmonasterium et rivulis in ericeto Hounsleiano observavit D. Doody. Herbarum caulibus foliis et aliis quisquiliis adnascitur". The use of the binomial "*Conferva reticulata*" followed by the name of the author is particularly interesting.

At the present day *Hydrodictyon* is regarded as a rare alga in Britain (Fritsch, 1935) and is apparently extremely sporadic in its appearance, so much so that were it not for these early records one would be tempted to regard it as merely an occasional visitor to the British Isles and not truly native. Probably, however, its rarity at the present day is attributable to changes in local conditions—it is more than probable that any search, no matter how thorough, would fail to reveal *Hydrodictyon* in either of the two localities mentioned by Doody! The true home of *H. reticulatum* would however appear to be regions which are liable to alternating periods of inundation and drought, particularly in warmer regions

such as the Nile lands of Egypt and paddy fields of China, where it seems that the alga appears regularly in vast quantities. It is widely distributed in the Northern Hemisphere—America, Europe, Asia and North Africa—but apart from the two records from near Grahamstown in South Africa (Heatherton Towers and a backwater on the Great Fish River) and one from near Sydney, N.S.W., Australia, it does not appear to have been recorded from the Southern Hemisphere. It is characteristic of the Northern warm temperate zone.

In the case of *H. patenaeforme* the occurrence is similar, except that the dry period is normally more prolonged and the periods of inundation less certain as well as of shorter duration and that it is on the whole native to warmer regions. The type of vegetative reproduction in the former species infers a more or less prolonged aquatic period before recourse must be had to the drought-resisting resting spores, whereas in the latter species although as shown here, the spores may germinate immediately should conditions be favourable to produce a fresh crop of nets, the alga is obviously not adapted for nor dependent on a prolonged aquatic existence. The pools in which it is found often dry up completely within a few weeks, though in other cases where it occurs in extensive vleis, such as Groen Vlei, the water may persist for several months. On the whole, it is characteristic of rather warmer regions than *H. reticulatum*, although there is considerable overlap in the geographical distribution. So far, besides the numerous records from South Africa (notably the Cape Flats, Kimberley, Grahamstown), it has been recorded from California (Stamford, University Campus, G. M. Smith), Utah (Seville Flowers), Texas (Brownsville, R. Runyon), in North America, and from Lake Titicaca (Tutin). A specimen at Kew from "Uruguay: ad Montevideo" labelled "*H. reticulatum* forma saepe gigantea" is certainly this species. It will probably prove to be even more widespread. The nets when fully mature are much coarser than those of *H. reticulatum*, where the common net, the daughter net, is composed of far more coenocytes which however never attain the size of those of *H. patenaeforme*.

H. africanum, though described 20 years earlier than *H. patenaeforme*, has still not been found anywhere outside a very restricted area in the Cape Province and is apparently confined to a narrow coastal strip extending about 100 miles north of the Cape Flats where it was originally found.

SUMMARY.

1. In *Hydrodictyon patenaeforme* the mature net produces swarmers which are always liberated into the water.

2. Such swarmers may either behave as gametes, conjugating to form a planozygote which settles down to form a hypnospor, or as accessory cells which settle down similarly but without conjugating.

3. Despite considerable variation in size it has not been possible to demonstrate that there are two distinct kinds of swarmers and the conclusion has been reached that all are facultative gametes which may also function as accessory cells, or zooids.

4. In *H. reticulatum* on the other hand two distinct types of swarmers are liberated, the smaller are gametes and conjugate; the larger are similar in size and structure to net-forming zooids and may function either as gametes or as accessory cells; the latter settle down without conjugation and develop into hypnosporos.

5. In *H. africanum* one type of motile swarmer is liberated, but two sizes occur, normally behaving as gametes, conjugating to form hypnosporos. The later experiments suggest that they may sometimes act as accessory spores, but confirmation of this is still needed.

6. In sexual reproduction the gametes of all three species are very similar in structure and size, but whereas *H. reticulatum* and *H. patenaeforme* are isogamous, *H. africanum* is anisogamous.

7. In all the species the hypnospor, no matter what its origin, having secreted a wall may either (i) undergo a period of growth followed immediately by germination, (ii) after a period of growth enter a more or less prolonged period of rest, or (iii) without preliminary growth enter a prolonged resting state eventually followed by growth prior to germination.

8. In *H. reticulatum* and *H. patenaeforme* the hypnosporos on germination produce comparatively large zoosporos, one in the case of azygosporos, in the case of zygosporos typically four or if large eight, but sometimes two, three, five, six or seven.

9. After a brief period of motility the zoospor settles down and forms a polyhedron.

10. The polyhedron in turn undergoes a period of growth culminating in division to form zooids which unite to form the germ net within a vesicle formed from the polyhedron wall or more usually from the inner part thereof.

11. In *H. africanum*, although polyhedra have occasionally been seen, the zoospor and polyhedral stage are usually cut out of the life cycle. After a period of growth the spore, after "hatching" by rupturing the outer wall, may either germinate to form a germ net directly, or undergo

further periods of growth each followed by "hatching", before producing the germ net.

12. In *H. patenaeforme* and *H. africanum* the germ net, most frequently formed of 512, 1,024 or more coenocytes, is typically flat and is the only net in the life cycle: it is comparatively long lived, reaching a large size, eventually producing swarmers. In both there is a tendency for the mature coenocytes to dissociate, much more markedly so in *H. africanum*, where the separate coenocytes are spherical and develop independently for a considerable time.

13. In *H. reticulatum* the germ net is fewer celled, most often a hollow sac, more rarely basket shaped, or flat with more or less double centre, or when few celled occasionally quite flat; it matures rapidly and produces cylindrical daughter nets, which soon constitute the entire and very large population, each consisting of thousands of coenocytes.

14. Before a coenocyte in any species divides it passes through the same sequence of changes, resulting in the division of the chloroplast into numerous small rounded plastids, disappearance of the pyrenoids, multiplication of the nuclei and rearrangement of the plastids to form a sheath round each nucleus, and finally cleavage of the protoplast into small polygonal uninucleate prisms—the pavement stage.

15. In *H. reticulatum* in a normal well nourished coenocyte in daughter-net formation, the following stages are seen:

- (i) Increase in size of the coenocyte already begun continues, partly due to the swelling of the wall; combined with a slight shrinkage of the central vacuole, the space between the wall and the vacuolar membrane in which the protoplast lies is consequently enlarged.
- (ii) Each segment of the pavement begins to round off changing its shape, and a colourless apex from which the flagella begin to emerge forms to one side.
- (iii) As the zooids are differentiated they move slightly apart from one another. Movement begins as a rather convulsive shivering motion, at first very faint.
- (iv) The two flagella lengthen but are always less than body length, and movement strengthens, the zooids beginning to move freely among one another, but always in a single peripheral layer. All protoplasmic connection between them has now disappeared.
- (v) Although confined between the outer protoplasmic membrane and the vacuolar membrane, the zooids do not adhere to either; but the latter prevents them from moving inwards towards the centre of the coenocyte.

- (vi) The zooids jostle one another as though elbowing their neighbours and pushing against them, thus increasing the space between them while contact between neighbours grows closer.
- (vii) The antero-posterior axis is now shorter than the equatorial axis parallel to the surface of the coenocyte. At each end of the latter axis short processes are thrown out towards two neighbours, most often three zooids uniting at each intersection, though the number may vary from two to several.
- (viii) As the ends of the axis unite, they are pushed out toward one another, the zooid twisting back and forth on this axis in the effort to complete union, helped by the action of the flagella to one side of the mesh which is beginning to form.
- (ix) As union is achieved, the flagella are withdrawn and movement ceases, contractile vacuoles continuing to pulsate for a time.
- (x) A wall is secreted round each zooid, the chloroplasts unite to form an incomplete median girdle, first one then other pyrenoids appear, the nuclei multiply, and the chloroplast extends, gradually lining the whole wall, while the coenocyte thus formed elongates, becoming first fusiform then cylindrical.
- (xi) Throughout the process of net formation the vacuolar membrane persists, thus keeping the zooids in the peripheral position. Eventually the outer wall is ruptured and the young net escapes; the vacuolar membrane finally disappears.

16. In all three species the germ net is formed in a basically similar fashion, allowing for (a) the much smaller size, (b) the shape of the comparatively large vesicle and (c) the much smaller number of zooids. Here again the vacuolar membrane plays an important rôle in the early stages of net formation. In all, the young coenocytes become cylindrical, but in *H. africanum* swelling soon after begins and the coenocytes becomes more or less spherical.

17. The dominant phase is haploid; the diploid phase is confined to the zygote (planozygote and zygospore) reduction division where known occurring at the germination of the zygospore.

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work done on that species is due. Her record is all the more valuable in that this species was actually growing in the same place as *H. patenae-forme*. To Miss E. L. Stephens I owe my first introduction to many of the vleis on the Cape Flats as well as a number of records of *H. africanus*. I am indebted to Professor Alan Burgess, formerly of Sydney University, for introducing me to "Captain Cook's stream of fresh water", where we found *H. reticulatum*, and to Dr. Margery Knight for much valuable assistance at various times. Finally, I should like to record my most grateful thanks to the heads of the Botany Departments of the University of Cape Town and of Rhodes University, Grahamstown, where much of this work was done.

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